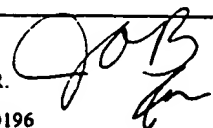# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/28592

A. CLASSIFICATION F SUBJECT MATTER

IPC(7) :Please See Extra Sheet.
US CL : 435/69.1, 243, 252.3, 252.33, 254.2, 320.1, 325, 410; 536/23.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 243, 252.3, 252.33, 254.2, 320.1, 325, 410; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ------ Y	US 5,416,008 A (BAILEY et al) 16 May 1995, whole document, particularly columns 4-6.	1-4, 8-10 -------- 5-7,11-26
Y,P	US 5,989,910 A (MERMOD et al) 23 November 1999, whole document.	1-26
Y	DEDHIA et al. Design of expression systems for metabolic engineering: coordinated synthesis and degradation of glycogen. Biotechnology and Bioengineering. 20 July 1997, Vol. 55, No. 2, pages 420-426, whole document.	1-26

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

*   Special categories of cited documents:

"A"   document defining the general state of the art which is not considered to be of particular relevance

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"O"   document referring to an oral disclosure, use, exhibition or other means

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"T"   later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&"   document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 FEBRUARY 2000	21 MAR 2000

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SHOCKETT et al. Diverse strategies for tetracycline-regulated inducible gene expression. Proceedings of the National Academy of Sciences, USA. May 1996, Vol. 93, pages 5173-5176, whole document.	1-26

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

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B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

EAST, MEDLINE, EMBASE, BIOSIS, CAPLUS
search terms: author's names, expression, vector, construct, autoregulated, cross-regulated, promoter, inducible, regulatable, Wilfred Chen, Hermann Bujard

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(54) Title: ANTIBIOTIC-BASED GENE REGULATION SYSTEM

(57) Abstract

The invention relates to a novel system for gene regulation in eukaryotic cells, and methods of using the same for protein production, tissue engineering and gene therapy. In particular, the invention provides a new system for antibiotic-regulated gene expression in eukaryotic cells based on sequences from *Actinomycetes* antibiotic resistance promoters, polypeptides that bind to the same in an antibiotic responsive manner, and nucleotides encoding such polypeptides. Further, the invention provides novel and sensitive methods of screening for candidate antibiotics.

Antibiotic-Based Gene Regulation System

Field of the Invention

The invention relates to a novel system for gene regulation in eukaryotic cells, and
5   methods of using the same for protein production. In particular, the invention provides a
new system for antibiotic-regulated gene expression in eukaryotic cells based on sequences
from *Actinomycetes* antibiotic resistance promoters, polypeptides that bind to the same in an
antibiotic responsive manner, and nucleotides encoding such polypeptides.

10   Background of the Invention

Controlled expression of transgenes or of target endogenous genes will likely be
essential for success of many genetic therapies. Constitutive expression of transgenes has
resulted in down-regulation of effector systems and/or cellular toxicity in animal studies
(Efrat *et al.*, 1995, Proc. Natl. Acad. Sci. USA 92, 3576-3580). Regulated expression in
15   response to metabolic, hormonal, or environmental signals is the normal situation for many
eukaryotic genes. In order to mimic natural physiological expression patterns with
transgenes, and to minimize interactions with human gene regulation signals, binary
promoter/transactivator configurations of heterologous origin which respond to
heterologous stimuli have been developed in recent years. However, many exogenous
20   stimuli which modulate these artificial mammalian regulons have proven to be incompatible
with human therapeutic use due to cytotoxicity or undesired side effects (Baim *et al.*, 1991,
Proc. Natl. Acad. Sci. USA 88, 5072-5076; Braselmann *et al.*, 1993, Proc. Natl. Acad. Sci.
USA 90, 1657-1661; No D. *et al.*, 1996, Proc. Natl. Acad. Sci. USA 93, 3346-3351; Rivera
et al., 1996, Nat. Medicine 2, 1028-1032; Suhr *et al.*, 1998, Proc. Natl. Acad. Sci. USA 95,
25   7999-8004; Wang *et al.*, 1994, Proc. Natl. Acad. Sci. USA 91, 8180-8184). The
tetracycline-regulated mammalian expression system avoids these problems, and is
described in U.S. Patent Nos.: 5,888,981; 5,866,755; 5,789,156; 5,654,168; and 5,650,298,
to name just a few examples. However, the tetracycline-regulated system can fail to
suppress gene expression adequately under repressed conditions.
30   Moreover, future gene therapy strategies will require technology which allows
independent control of two different transgenes or sets of transgenes which are
cotranscribed in a multicistronic configuration. For example, many tissue expansion and *ex
vivo* gene therapy scenarios will require a two-step process beginning with expression of
growth-promoting genes to enable expansion of grafted tissues in culture, followed by
35   induction of growth suppressors to prevent tumorigenic behavior of treated cells after
reimplantation. Sustained proliferation control is also required for stem cell-based

technologies currently evaluated for eventual cell and tissue replacement therapy, since stem cells are tumorigenic (Rossant *et al.*, Nat. Biotechnol. 17, 23-24; Solter *et al.*, Science 283, 1468-1470). The second independent gene regulation system could be used in such cells for pharmacologic control of one or several secreted therapeutic proteins, such as insulin, to

5   enable titration of circulating proteins into the therapeutic range or adapt expression to optimal daily dosing regimes. There is, therefore, a need for new mammalian gene regulation systems which employ modern, therapeutically proven antibiotics as controlling agents, and which can be used in combination with the tetracycline regulation system, with minimal interaction between tetracycline control and the new control modality.

10   The human oral antibiotic pristinamycin consists, like other streptogramins, of a mixture of two structurally dissimilar molecules, the streptogramin A component pristinamycin II (PII), a polyunsaturated macrolactone, and the streptogramin B component pristinamycin I (PI), a cyclic hexadepsipeptide. The water-soluble form of pristinamycin, Synercid˙, recently approved in the U.S. and Europe for use against most multiple drug-

15   resistant Gram-positive bacteria (Barriuere *et al.*, 1994, Expert Opin. Invest. Drugs 3, 115-131; Baquero *et al.*, 1997, J. Antimicrob. Chemother. 39, 1-6), is composed of dalfopristin, a 26-sulphonyl derivative of PII, and quinupristin, which is derived from PI by synthetic addition of a (5δ R)-[(3S)-quinuclidinyl] thiomethyl group. Virginiamycin is another important streptogramin used as a growth promotant in livestock feed (Nagaraja *et al.*, 1998,

20   J. Anim. Sci. 76, 287-298). Either the A or B streptogramin components are individually bacteriostatic, but streptogramins A and B together are synergistically bactericidal (up to 100 times more active), a phenomenon which lowers incidence of acquired antibiotic resistance, since high level resistance to the combined streptogramins is likely only when both type A and type B streptogramin resistance determinants are present simultaneously

25   (Cocito *et al*, 1997, J. Antimicrob. Chemother. 39, 7-13). Recently, a pristinamycin resistance determinant (*ptr*) has been cloned from *S. pristinaespiralis* (Blanc *et al.*, 1995, Mol. Microbiol. 17, 989-999; Salah-Bey *et al.*, 1995, Mol. Microbiol. 17, 1001-1012; Salah-Bey *et al.*, 1995, Mol. Microbiol. 17, 1109-1119). Expression from the *ptr* promoter (containing a recognition sequence termed P_{PTR}) is induced by pristinamycin, particularly by

30   PI. A protein called Pip (pristinamycin-induced protein) was identified based on its affinity to P_{PTR} in gel retardation experiments (Salah-Bey *et al.*, 1995, Mol. Microbiol. 17, 1109-1119).

Recently, the prevalence of multidrug resistant human pathogenic bacteria has increased dramatically. This increase correlates with an escalation of bacterial disease and

35   related mortality. Also, antibiotic chemotherapy is becoming more difficult as the

percentage of elderly and immunocompromised patients grows. The European Commission
has already reacted to this situation by banning the use of certain antibiotics as a growth
promoter in livestock feed, among them the streptogramin virginiamycin, so as to limit the
environmental spread of antibiotics (thought to be a major driving force for selection of

5   multidrug resistant pathogenic bacteria) thereby preserving the use of antibiotics for human
therapy.

Summary of the Invention

The present invention is directed to a new system for antibiotic-regulated gene

10   expression in eukaryotic cells based on sequences from *Actinomycetes* antibiotic resistance
promoters, and polypeptides that bind to the same in an antibiotic responsive manner. In
one aspect, the invention relates to an isolated nucleic acid encoding a polypeptide which
binds to a P_{abr} sequence in the absence of its cognate antibiotic. The P_{abr} sequence is derived
from an actinomycetes bacterium, in particular an antibiotic responsive operon from an

15   actinomycetes bacterium. In another aspect, the invention relates to an isolated nucleic acid
encoding a fusion protein which regulates transcription, the fusion protein having a first
polypeptide which binds to a P_{abr} sequence in the absence of its cognate antibiotic,
operatively linked to a second polypeptide which activates or represses transcription in
eukaryotic cells. In another aspect, the invention relates to the proteins encoded by such

20   nucleic acids. These proteins can be used to activate or repress transcription from a desired
nucleotide sequence to be transcribed that is operatively linked to a P_{abr} sequence.

The invention also includes host cells that contain nucleic acids encoding the
proteins and fusion proteins of the invention. Such host cells optionally contain a desired
nucleotide sequence to be transcribed operatively linked to a P_{abr} sequence. The nucleotide

25   sequence to be transcribed can be endogenous or exogenous to the host cell.

Yet another aspect of the invention provides an isolated nucleic acid having a P_{abr}
sequence operatively linked to a first eukaryotic promoter. The first eukaryotic promoter
can also be operatively linked to a first coding sequence. Optionally, the nucleic acid can
also contain at least one tet operator sequence operatively linked to a second eukaryotic

30   promoter, which in turn can be operatively linked to a second coding sequence. Either
coding sequence can encode any protein of interest for which production is desired. Further,
one of the coding sequences can encode a tumor suppressor gene product or a gene product
which activates cell proliferation. Still further, at least one of the promoters can be

35   operatively linked to more than one coding sequence through the use of, for example, an
internal ribosomal entry site. Host cells genetically engineered to contain these nucleic

acids are also provided by the invention. By "genetically engineered" is meant that the host
cells are manipulated by molecular biology techniques, including transformation,
transfection, and recombination (such as homologous or non-homologous recombination),
to contain the desired nucleic acid.

5   In another aspect, the invention provides a method for regulating expression of an
P_{abr} -linked gene in a eukaryotic cell. The method entails introducing into the cell a nucleic
acid molecule encoding a PIT. The PIT can be a P_{abr} -binding protein, or can comprise a P_{abr}
-binding protein operably linked to a polypeptide that activates or represses transcription in
eucaryotic cells, thereby rendering the P_{abr} -linked gene capable of regulation by an

10 antibiotic that binds to the P_{abr} -binding protein in the cell. One can then modulate the level
of the antibiotic in the cell to regulate expression of the P_{abr} -linked gene

          Still another aspect of the invention is a process for producing a protein by culturing
a eukaryotic cell containing a P_{abr} -linked gene that encodes the protein and a nucleic acid
molecule encoding a PIT. The PIT can be a P_{abr} -binding protein, or can comprise a P_{abr} -

15 binding protein operably linked to a polypeptide that activates or represses transcription in
eucaryotic cells. Expression of the P_{abr} -linked gene is then regulated by modulating the
level of an antibiotic that binds to the P_{abr} -binding protein in the cell. Optionally, the
process entails the step of collecting the protein produced by the cell.

          Another aspect of the invention is a method of screening for candidate antibiotics.
20 The method entails incubating the host cells of the invention, the host cells containing a P_{abr}
-linked reporter gene and a sequence encoding a P_{abr} -binding protein, in the presence of a
test compound, wherein a change in the transcription of the reporter gene indicates that the
test compound is a candidate antibiotic.

25
Brief Description of the Figures

          Figure 1: Gel retardation assay showing Pip/P_{PTR} interaction. P_{PTR} of $S.$
pristinaespiralis contains 3 dyad symmetrical binding sites (lane A; P_{PTR} in the absence of
Pip) all of which are saturated at relatively large amounts of purified Pip (0.4 pmol; lane A)
resulting in a single shifted band. Smaller amounts of Pip (0.02 pmol) presented an
30
intermediate situation in which all possible permutations of site occupancy are represented
in three distinct retarded bands (lane C). Pip (0.2 pmol lanes D-M) was titrated with a range
of PI from 50 pmol to 0.01 pmol (lanes D-H; D: 50 pmol; E: 10 pmol; F: 1 pmol; G: 0.1
pmol; H: 0.01 pmol). Under identical conditions the semisynthetic PI derivative
quinupristin was also titrated (lanes I-M; I: 50 pmol; J: 10 pmol; K: 1 pmol; L: 0.1 pmol; M:
35
0.01 pmol).

Figure 2: Dose-response curve for PI-dependent gene expression. The cell line CHO-PIT1-SEAP was grown for 48 h at different PI concentrations (ng/ml). The relative SEAP production is shown over 5 orders of magnitude of PI concentration.

Figure 3: Graphical representation of pMF188, a vector that combines tetracycline-responsive SEAP (human secreted alkaline phosphatase) and pristinamycin-responsive GFP expression in a single double regulated expression unit: $P_{hCMV*-1}$-SEAP-pA_I-P_{PIR}-GFP-pA_{II}.

Figure 4: Graphical representation of pMF201, a vector that contains tet-regulated CFP (cyan fluorescent protein) and pristinamycin-regulated YFP (yellowish fluorescent protein) in a single expression unit: $P_{hCMV*-1}$-CFP-pA_I8 -P_{PIR}-YFP-pA_{II}.

Figure 5: Graphical representation of pMF196, a vector that is isogenic to pMF188 and pMF201 but contains the following double regulated expression unit: P_{PIR}-YFP-pA_I-$pA_{II}s28$ -CFP-$P_{hCMV*-1}$.

Figure 6: Graphical representation of vector pDuoRex1. pDuoRex1 contains two expression units which allow independent adjustable gene expression in mammalian cells using tetracycline and pristinamycin as regulating agents. The tetracycline-responsive expression unit is driven by $P_{hCMV*-1}$ followed by an internal ribosomal entry site (IRES) flanked by two multiple cloning sites. The expression unit contains a terminal artificial polyadenylation sequence (pA1). The IRES element enables dicistronic $P_{hCMV*-1}$-driven expression. The second regulatable cistron is driven by P_{PIR} followed by a multiple cloning site and terminated by a second polyadenylation sequence.

Figure 7: Graphical representation of vector pMF195. PMF195 is a pDuoRex1 derivative containing the tumor suppressor gene p27 under control of the tetracycline-responsive promoter $P_{hCMV*-1}$ and the Mek1 gene under control of P_{PIR}. Mek1 is a central kinase of the main growth factor signalling pathway and was particularly mutated (Mek1DD) to be constitutively active even in the absence of exogenous growth factors. Mek1 is an example of a class of proteins which promote cell proliferation.

Figure 8: Graphical representation of vectors pMF189 and pMF229.

Figure 9: Graphical representation of multicistronic vectors. Figure 9A illustrates vectors pTRIDENT9 and pTRIDENT10, while Figure 9B illustrates pTRIDENT11 and pTRIDENT12. The pTRIDENT-derived mammalian expression vectors contain a tricistronic expression unit which is either driven by the pristinamycin-repressible (P_{pir}) or the pristinamycin-inducible (P_{pir}ON) promoter. Whereas the first cistron of the tricistronic expression unit is transcribed in a cap-dependent manner, the subsequent genes rely on cap-independent translation initiation based on internal ribosomal entry sites of polyioviral

(IRES) origin or of encephalomyocarditis virus. The multicistronic expression unit is terminated by a polyadenylation site (pA) of the SV40 virus.

Detailed Description of the Invention

5   Systems to control expression of foreign genes will be essential tools for future cell- and gene-based human therapies. The present invention is directed to a new system for antibiotic-regulated gene expression in eukaryotic cells based on sequences from *Actinomycetes* antibiotic resistance promoters, and polypeptides that bind to the same in an antibiotic responsive manner.

10   For purposes of the invention, a P_{abr} sequence is a sequence from an *Actinomycetes* antibiotic resistance promoter that binds a particular polypeptide, AIP (also referred to herein as a "polypeptide which binds to a P_{abr} sequence in the absence of its cognate antibiotic"), in an antibiotic dependent way. The antibiotic resistance promoter can be derived from a naturally-occurring episome, but is preferably a chromosomal promoter.

15 Preferably, AIP binds to the P_{abr} sequence in the absence of its cognate antibiotic, and is released from the P_{abr} sequence when antibiotic is present, although the reverse situation is also within the scope of the invention. Accordingly, in the presence of the AIP, expression from the antibiotic resistance promoter containing the P_{abr} sequence is regulated by the presence or absence of antibiotic. Thus, for purposes of the invention, the term "cognate

20 antibiotic" means the antibiotic which when bound to the AIP results in the release of the protein from its P_{PIR} binding site.

        AIPs are, for the purposes of the invention, derived from or related to AIP proteins produced by *Actinomycetes*. By "derived from" AIP proteins produced by *Actinomycetes*, is

25 meant, in this context, that the amino acid sequence is identical to a naturally occurring AIP, or contains only conservative amino substitutions and but remains at least 70%, preferably 80%, and more preferably 90% identical at the amino acid level. By "related to" AIP proteins produced by *Actinomycetes* is meant, for purposes of the invention, that the polynucleotide sequence that encodes the amino acid sequence hybridizes to a naturally

30 occurring AIP produced by *Actinomycetes* under at least low stringency conditions, more preferably moderate stringency conditions, and most preferably high stringency conditions, and binds to a P_{abr} recognition sequence. Conservative substitutions known in the art and described by Dayhof, M.D., 1978, Nat. Biomed. Res. Found., Washington, D.C., Vol. 5, Sup. 3, among others. Genetically encoded amino acids are generally divided into four

35 groups: (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine; (3) non-

polar = alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar = glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan and tyrosine are also jointly classified as aromatic amino acids. A substitution of one amino acid in a particular group with another amino acid

5   in the same group is generally regarded as a conservative substitution.

The *Actinomycetes* include Gram-positive bacteria from the following taxonomic groups: Actinomycineae; Corynebacterineae; Frankineae; Glycomycineae; Kineococcus group; Micrococcineae; Micromonosporineae; Propionibacterineae; Pseudonocardineae; Streptomycineae; Streptosporangineae; and unclassified Actinomycetales (Stackebrandt *et*

10 *al.*, 1997, Int. J. Syst. Bacteriol. 47 (2): 479-491). Additional information regarding *Actinomycetes*, and the members of each group, can be obtained from the National Center for Biotechnology at http://www.ncbi.nlm.nih.gov taxonomy collection. Preferred actinomycetes are streptomycetes, and are thus from the group Streptomycineae.

The invention is illustrated below in one embodiment using a particularly preferred

15 antibiotic system employing streptogramins and the Pip/P_{PTR} interaction. Specifically, one particularly preferred AIB is the Pip of *S. coelicolor* binds to a particular P_{abr}, the P_{PTR}. recognition site in an antibiotic responsive manner. Thus the invention provides, in one aspect, the complete nucleotide coding sequence of the Pip protein (SEQ ID NO:29), and the deduced amino acid sequence of Pip. The amino acid sequence of Pip was used in

20 protein database searches to identify additional *Streptomyces* and *Amycolatopsis* genes within antibiotic biosynthetic clusters bearing sequence similarity to Pip that may respond to other antibiotics. In particular, Pip contains a helix-turn-helix motif characteristic of DNA binding proteins. A computer search was conducted for proteins containing this motif encoded within antibiotic biosynthetic gene clusters which also encode a transmembrane

25 protein antibiotic resistance gene. Results are shown the following Table 1. These actinomycetes, as well as others yet to be discovered or identified, can contain P_{abr} sequences and AIPs that can be used in the compositions and methods of the invention.

Table 1: *Sreptomyces* and *Amycolatopsis* antibiotic biosynthetic gene clusters.

Protein	%AA identity to Pip	Putative antibiotic inducer	Clinical approval	Actinomycete
pip	100	pristinamycin I (confirmed)	+	*Streptomyces coelicolor*
rifQ	49	rifamycin	+	*Amycolatopsis mediterranei* (1)
orfY	<25	rapamycin	+	*Streptomyces hygroscopicus* (5)

35

dnrO	<25	daunorubicin (adriamycin)	+	Streptomyces peucetius (4)
actII (orf1)	30	actinorhodin	-	Streptomyces coelicolor (2)
tcmR	<25	tetracenomycin (confirmed)	- (?)	Streptomyces glaucescens (3)
lanK	<25	landomycin	- (?)	Streptomyces cyanogenus (6)

References for TABLE 1 are as follows:

1. August et al., 1998, Biosynthesis of the ansamycin antibiotic rifamycin: deductions from the molecular analysis of the rif biosynthetic gene cluster of Amycolatopsis mediterranei S699. Chem. Biol. 5:69-79.

2. Fernandez et al., 1991, The act cluster contains regulatory and antibiotic export genes, direct targets for translational control by the bldA tRNA gene of Streptomyces. Cell 66:769-780.

3. Guilfoile et al., 1992, Sequence and transcriptional analysis of the Streptomyces glaucescens tcmAR tetracenomycin C resistance and repressor gene loci. J Bacteriol 174:3651-8.

4. Otten et al., 1995, Regulation of daunorubicin production in Streptomyces peucetius by the dnrR2 locus. J. Bacteriol. 177:1216-1224.

5. Schwecke et al., 1995, The biosynthetic gene cluster for the polyketide immunosuppressant rapamycin. Proc Natl Acad Sci U S A 92:7839-7843.

6. Westrich et al., 1999, Cloning and characterization of a gene cluster from Streptomyces cyanogenus S136 probably involved in landomycin biosynthesis. FEMS Microbiol Lett 170:381-7.

Actinomycetes antibiotic-resistant promoters that contain P_{abr} sequences can also be identified by generating Actinomycetes gene libraries in a heterologous host, and growing the heterologous host under conditions containing selective antibiotics. Emerging resistant clones containing antibiotic resistant determinants can be identified by their characteristic protein binding motifs following sequence analysis.

Polynucleotide sequences encoding the first polypeptide which binds to a P_{abr} sequence in the absence of its cognate antibiotic can be used to clone homologous AIPs in other actinomycetes. Such homologous AIPs can also be used as the first polypeptide of the fusion protein, and their P_{abr} recognition sequence operably linked to the eukaryotic promoter of interest. Thus, the invention also relates to nucleic acids hybridizable to or

complementary to nucleotides which encode the AIPs described herein. A preferred AIP is the repressor of a streptogramin resistance operon of *S. coelicolor*, Pip, described more fully below. Such AIPs are at least 50%, preferably 60%, more preferably 70%, even more preferably 80%, yet more preferably 90%, and most preferably 95% homologous at the

5 amino acid sequence level to an AIP described herein Homology can be calculated using, for example, the BLAST computer program, version 2.0, available on the World-Wide Web at http://www.ncbi.nlm.nih.gov. Typical parameters for determining the similarity of two sequences using BLAST 2.0 are a reward for match of 1, penalty for mismatch of -2, open gap and extension gap penalties of 5 and 2, respectively, a gap dropoff of 50, and a word

10 size of 11.

In a specific embodiment, a nucleic acid that is hybridizable to an AIP nucleic acid, or to a nucleic acid encoding an AIP amino acid, under conditions of low stringency is provided. Procedures using such conditions of low stringency are as follows (*see also* Shilo and Weinberg, 1981, Proc. Natl. Acad. Sci. USA 78:6789-6792): Filters containing DNA

15 are pretreated for 6 h at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 μg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution with the following modifications: 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 μg/ml salmon sperm DNA, 10% (wt/vol) dextran sulfate, and 5-20 X 10^6 cpm ^{32}P-labeled probe is used. Filters

20 are incubated in hybridization mixture for 18-20 h at 40°C, and then washed for 1.5 h at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS. The wash solution is replaced with fresh solution and incubated an additional 1.5 h at 60°C. Filters are blotted dry and exposed for autoradiography. If necessary, filters are washed for a third time at 65-68°C and reexposed to film. Other conditions of low

25 stringency which can be used are well known in the art (*e.g.*, as employed for cross-species hybridizations).

In another specific embodiment, a nucleic acid that is hybridizable to an AIP nucleic acid, or to a nucleic acid encoding an AIP amino acid, under conditions of moderate stringency is provided. Procedures using such conditions of moderate stringency are as

30 follows: Filters containing DNA are pretreated for 6 h at 55°C in a solution containing 6X SSC, 5X Denhart's solution, 0.5% SDS and 100 μg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution and 5-20 X 10^6 cpm ^{32}P-labeled probe is used. Filters are incubated in hybridization mixture for 18-20 h at 55°C, and then washed twice for 30 minutes at 60°C in a solution containing 1X SSC and 0.1% SDS. Filters are

35 blotted dry and exposed for autoradiography. Other conditions of moderate stringency

which can be used are well-known in the art. Washing of filters is done at 37°C for 1 h in a solution containing 2X SSC, 0.1% SDS.

In another specific embodiment, a nucleic acid that is hybridizable to an AIP nucleic acid, or to a nucleic acid encoding an AIP amino acid, under conditions of high stringency is

5   provided. Procedures using such conditions of high stringency are as follows: Prehybridization of filters containing DNA is carried out for 8 h to overnight at 65°C in buffer composed of 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 μg/ml denatured salmon sperm DNA. Filters are hybridized for 48 h at 65°C in prehybridization mixture containing 100 μg/ml denatured salmon sperm

10   DNA and 5-20 X 10^6 cpm of ^{32}P-labeled probe. Washing of filters is done at 37°C for 1 h in a solution containing 2X SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA. This is followed by a wash in 0.1X SSC at 50°C for 45 min before autoradiography. Other conditions of high stringency which can be used are well known in the art.

New AIP proteins can also be isolated by binding to P_{abr} sequences. For example,

15   the polynucleotide with the P_{plr} sequence is immobilized on a matrix and ideally packed in a column. Bacterial extracts are applied to the column under conditions which allow PIP homologs to bind to the immobilized target sequence. Following appropriate washing steps, the PIP homolog is eluted by suitable conditions (e.g., addition of antibiotic) and the sequence of the purified protein determined and the corresponding gene cloned.

20   The invention also includes isolated or purified AIP proteins and polypeptides of the invention. AIP proteins of the invention include polypeptides that bind to P_{abr} sequences, as well as fusion proteins containing a first polypeptide that binds to P_{abr} sequences operatively linked to a second polypeptide which activates or represses transcription in eukaryotic cells. In this context, operatively linked means that the two proteins are covalently or non-

25   covalently bound to one another in such a manner that they retain their functional activities of binding to P_{abr} sequence (first polypeptide) and activating or repressing transcription (second polypeptide).

As used herein, a polypeptide is "substantially purified" where the polypeptide constitutes the majority (i.e., at least about 50%) by weight of the material in a particular

30   preparation. Also, as used herein, a polypeptide is "isolated" where the polypeptide constitutes at least about 90 wt% of the material in a particular preparation.

The fusion proteins of the invention contain both the first polypeptide which binds to a P_{abr} sequence in the absence of its cognate antibiotic, and a second polypeptide which

35   activates or represses transcription in eukaryotic cells. By activating transcription is meant

that the rate of transcriptional initiation is increased from the nucleotide sequence to be transcribed that is operatively linked to a P_{abr} sequence when the fusion protein that activates transcription is bound to the P_{abr} sequence, as opposed to when it is not bound. Similarly, by repression of transcription is meant that the rate of transcriptional initiation is decreased

5   from the nucleotide sequence to be transcribed that is operatively linked to a P_{abr} sequence when the fusion protein that represses transcription is bound to the P_{abr} sequence, as opposed to when it it not bound.

Accordingly, in one aspect, the first polypeptide of the fusion protein that activates transcription is operatively linked to a second polypeptide which directly or indirectly

10   activates transcription in eukaryotic cells. To operatively link the first and second polypeptides, typically nucleotide sequences encoding the first and second polypeptides are ligated to each other in-frame to create a chimeric gene encoding a fusion protein, although the first and second polypeptides can be operatively linked by other means that preserve the function of each polypeptide (*e.g.*, chemically crosslinked). The first and second

15   polypeptides can be in any order. The second polypeptide of the transactivator can itself possess transcriptional activation activity (*i.e.*, the second polypeptide directly activates transcription) or it activates transcription by an indirect mechanism, through recruitment of a transcriptional activation protein to interact with the fusion protein. Accordingly, the term "a polypeptide which activates transcription in eukaryotic cells" includes polypeptides

20   which either directly or indirectly activate transcription.

Polypeptides which can function to activate transcription in eukaryotic cells are well known in the art and are described, for example, in U.S. Patent No. 5,654,168. Such polypeptides include the herpes simplex virus virion protein 16 (VP16, the amino acid sequence of which is disclosed in Triezenberg, S. J. *et al.*, 1988, Genes Dev. 2:718-729),

25   particularly the 127 amino acid C-terminus or the 11 amino acid C-terminus. Suitable C-terminal peptide portions of VP16 are described in Seipel, K. *et al.* (EMBO J., 1992, 13:4961-4968).

Acidic transcription activation domains, proline-rich transcription activation domains, serine/threonine-rich transcription activation domains and glutamine-rich

30   transcription activation domains can all be used in the compositions and methods of the invention. VP16 polypeptides and amino acid residues 753-881 of GAL4 are acidic activating domains. Another polypeptide that activates transcription is the p65 domain of NF-κB (Schmitz and Baeuerle, 1991, EMBO J. 10:3805-3817). Examples of proline-rich activation domains include amino acid residues 399-499 of CTF/NF1 and amino acid

35   residues 31-76 of AP2. Examples of serine/threonine-rich transcription activation domains

include amino acid residues 1-427 of ITF1 and amino acid residues 2-451 of ITF2.
Examples of glutamine-rich activation domains include amino acid residues 175-269 of
Oct1 and amino acid residues 132-243 of Spl. The amino acid sequences of each of the
above described regions, and of other useful transcriptional activation domains, are

5   disclosed in Seipel, K. *et al.* (EMBO J., 1992, 13:4961-4968). This reference also
describes methods of identifying new transcriptional activation domains which are within
the scope of the invention.

In another embodiment, the second polypeptide of the fusion protein indirectly
activates transcription by forming a non-covalent association with a transcriptional

10   activator. For example, an AIP of the invention can be fused to a polypeptide domain (*e.g.*,
a dimerization domain) capable of mediating a protein-protein interaction with a
transcriptional activator protein, such as an endogenous activator present in a host cell.
Non-covalent interactions between DNA binding domains and transactivation domains are
known in the art (see *e.g.*, Fields and Song, 1989, Nature 340:245-247; Chien *et al.*, 1991,

15   Proc. Natl. Acad Sci. USA 88:9578-9582; Gyuris *et al.*,1993, Cell 75:791-803; and Zervos,
A. S., 1993, Cell 72:223-232). Examples of suitable interaction (or dimerization) domains
include leucine zippers (Landschulz *et al.*, 1989, Science 243:1681-1688), helix-loop-helix
domains (Murre, C. *et al.*, 1989, Cell 58:537-544) and zinc finger domains (Frankel, A. D.
et al., 1988, Science 240:70-73).

20   In another aspect, the polypeptide that binds P_{abr} can be used by itself to repress
transcription in the absence of antibiotic. In this manner, the polypeptide that binds P_{abr}
prevents transcription when bound to the P_{abr} sequence, presumably by interfering with
binding of activating transcription factors. In the absence of antibiotic, transcription from
the P_{abr} linked promoter is absent or minimal. When antibiotic is added, however, the

25 polypeptide that binds P_{abr} is released, thereby allowing transcription to occur.

In an alternative embodiment, the fusion protein that binds P_{abr} can be used to
repress transcription. In one aspect, the first polypeptide is operatively linked, as described
above, to a second polypeptide which directly or indirectly represses transcription in
eukaryotic cells. Proteins and polypeptide domains within proteins which can function to

30 repress transcription in eukaryotic cells have been described in the art (for reviews see, *e.g.*,
Renkawitz, R., 1990, Trends in Genetics 6:192-197; and Herschbach, B. M. and Johnson,
A. D., 1993, Annu. Rev. Cell. Biol. 9:479-509). Such domain can have a direct inhibitory
effect on the transcriptional machinery or can repress transcription indirectly by inhibiting
the activity of activator proteins. Accordingly, the term "a polypeptide that represses

35 transcription in eukaryotic cells" as used herein is intended to include polypeptides which

act either directly or indirectly to repress transcription. As used herein, "repression" of transcription is intended to mean a diminution in the level or amount of transcription of a target gene compared to the level or amount of transcription prior to regulation by the transcriptional inhibitor protein. Transcriptional inhibition may be partial or complete.

5   A transcriptional "repressor" or "silencer" domain as described herein is a polypeptide domain that retains its ability to repress transcription when the domain is transferred to a heterologous protein. Proteins which have been demonstrated to have repressor domains that can function when transferred to a heterologous protein include the v-erbA oncogene product (Baniahmad, A. *et al.*, 1992, EMBO J. 11:1015-1023) (*e.g.*,

10 approximately amino acid residues 362-632 of the native v-erbA oncogene product), the thyroid hormone receptor (Baniahmad, *supra*), the retinoic acid receptor (Baniahmad, *supra*), the Drosophila Krueppel (Kr) protein (Licht, J. D. *et al.*, 1990, Nature 346:76-79; Sauer, F. and Jackle, H., 1991, Nature 353:563-566; Licht, J. D. *et al.*, 1994, Mol. Cell. Biol. 14:4057-4066) (such as C64KR, which is amino acids 403-466 of the native protein,

15 or amino acids 26-110 of Kr), and the KRAB domain of the kox1 gene family (Deuschle *et al.*, 1995, Mol. Cell. Biol. 15:1907-1914). Other proteins which have transcriptional repressor activity in eukaryotic cells include the Drosophila homeodomain protein even-skipped (eve) (Han and Manley, 1993, Genes & Dev. 7: 491-503), the *S. cerevisiae* Ssn6/Tup1 protein complex (Herschbach and Johnson, *supra*), the yeast SIRI protein (see

20 Chien *et al.*, 1993, Cell 75:531-541), NeP1 (see Kohne *et al.*, 1993, J Mol. Biol. 232:747-755), the Drosophila dorsal protein (see Kirov *et al.*, 1994, Mol. Cell. Biol. 14:713-722; Jiang, *et al.*, 1993, EMBO J. 12:3201-3209), TSF3 (see Chen, *et al.*, 1993, Mol. Cell. Biol. 13:831-840), SF1 (see Targa, *et al.*, 1992, Biochem. Biophys. Res. Comm.. 188:416-423), the Drosophila hunchback protein (see Zhang, *et al.*, 1992, Proc. Natl. Acad.

25 Sci. USA 89:7511-7515), the Drosophila knirps protein (see Gerwin, *et al.*, 1994, Mol. Cell. Biol. 14:7899-7908), the WT1 protein (Wilm's tumor gene product) (see Anant, *et al.*, 1994, Oncogene 9:3113-3126; Madden *et al.*,, 1993, Oncogene 8:1713-1720), Oct-2.1 (see Lillycrop, *et al.*, 1994, Mol. Cell. Biol. 14:7633-7642), the Drosophila engrailed protein (see Badiani, *et al.*, 1994, Genes Dev. 8:770-782; Han and Manley,, 1993, EMBO J.

30 12:2723-2733), E4BP4 (see Cowell and Hurst,, 1994, Nucleic Acids Res. 22:59-65) and ZF5 (see Numoto, *et al.*, 1993, Nucleic Acids Res. 21:3767-3775).

          Non-limiting examples of polypeptide domains that can be used as silencing domains include: amino acid residues 120-410 of the thyroid hormone receptor alpha (THR.alpha.), amino acid residues 143-403 of the retinoic acid receptor alpha (RAR.alpha.),

35 amino acid residues 186-232 of knirps, the N-terminal region of WT 1 (see Anant, *supra*),

the N-terminal region of Oct-2.1 (see Lillycrop, *supra*), a 65 amino acid domain of E4BP4 (see Cowell and Hurst, *supra*) and the N-terminal zinc finger domain of ZF5 (see Numoto, *supra*). Moreover, shorter or longer polypeptide fragments encompassing these regions that still retain full or partial repression activity are also contemplated.

5   In addition to previously described transcriptional repressor domains, novel transcriptional repressor domains, which can be identified by standard techniques (*e.g.*, reporter gene constructs), are within the scope of the invention.

Construction of the nucleic acids of the invention can be accomplished by those of skill in the art using standard molecular biology techniques (*see*, for example, Maniatis *et*
10 *al.*, 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel *et al.*, 1989, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y.). Typically, manipulation and generation of nucleic acids is performed using prokaryotic host cells, but the invention also encompasses chemical synthetic methods of nucleic acid generation and manipulation.

15   The host cells for use in the methods and compositions of the invention can also be any eukaryotic cell such as mammalian cells, fungal cells, plant cells and microbial parasites. The invention is illustrating below by way of non-limiting examples using a variety of mammalian cells. However, other types of eukaryotic cells can also be used. For example, the tetracycline-regulatable promoter system has been adapted for use in yeast
20 (Gari *et al.*, 1997, Yeast 13:837-848); similarly, the methods and compositions of the invention can also be used in such cells. Methods of genetically engineering a host cell to contain the nucleic acids of the invention are well known to those of skill in the art and include transformation, transfection, and electroporation. The nucleic acids can be carried extrachromasomally or on the chromosome. Integration can be random, homologous, or
25 site-specific recombination. Culturing a host cell is understood to include both *in vitro* culture and *in vivo* culture (for example, growing eukaryotic cells in tissue culture, growing cells in a host organism such as by implantation in a body cavity or graft, removing cells from a particular individual and replacing them after genetically engineering the cells to contain the nucleic acids of the invention, *etc.*).

30   A P_{abr}-linked gene is defined herein as a promoter which directs the expression of a coding sequence, wherein the promoter and coding sequence are operatively linked to a P_{abr} sequence. Preferably, the promoter is a eukaryotic promoter. By operatively linked in this context is meant that the P_{abr} sequence is placed proximal to the promoter, either 5' to, or 3' to, or within the sequence of the promoter, such that when a fusion protein that modulates
35 transcription is bound to the P_{abr} sequence, initiation of transcription at the promoter is

affected. Particularly preferred P_{abr} sequences are those P_{ptr} sequences from *S. coelicolor* and *S. pristinaespiralis*; the use of these sequences to construct P_{abr} -linked genes are illustrated below by way of non-limiting examples.

5   The coding sequence operatively linked to the promoter can encode for any gene product for which regulated expression is desired, and can be exogenous to the host cell or endogenous. By endogenous coding sequence is meant coding sequence that is naturally present in the host cell and not introduced into the host cell via transformation techniques. Exogenous coding sequence is not endogenous coding sequence. Coding sequences include not only sequences encoding proteins, but also other coding sequences, *e.g.*, encoding

10   antisense gene products, ribozymes, etc. Further, the coding sequences can be multicistronic (*see*, for example, U.S. Application No. 08/948,381, filed October 9, 1997).

        Production of any gene product can be regulated using the compositions and methods of the present invention. For example, production of a marker gene product, such as green fluorescent protein, or of a model secreted gene product, such as SEAP, may be

15   regulated. Naturally, the invention finds particular use in the production of products of industrial or pharmaceutical interest such as industrial enzymes (*e.g.* proteases, cellulases, glycosidases, or ligninases), interferons (*e.g.* β-INF, α-INF, γ-INF), hGH, insulin, erythropoietin, tissue plasminogen activator (tPA), DNAse, monoclonal antibodies, Factor VIII, Factor VII, Factor IX, HSA, IL-2, glucagon, EGF, GCSF, GMCSF, thrombopoietin,

20   gp160, HbSAg, and other viral antigenic proteins and peptides (rotavirus, HIV, p53ras).

        Another aspect of the invention is the use of tumor suppressor gene products to regulate proliferation of the host cells of the invention. Regulated expression of tumor suppressor gene products are particularly useful for a variety of applications. For example, one may want the host cells to undergo a rapid proliferation phase followed by a production

25   phase where cellular energies are devoted to protein production, or a rapid proliferation phase *in vitro* followed by regulated growth *in vivo* (*see*, for example, U.S. Application No. 08/948,381, filed October 9, 1997, the disclosure of which is incorporated by reference). For purposes of the invention, tumor suppressor gene products are intracellular proteins that block the cell cycle at a cell cycle checkpoint by interaction with cyclins, Cdks or cyclin-

30   Cdk complexes, or by induction of proteins that do so. Thus, these tumor suppressor gene products inhibit the cyclin-dependent progression of the cell cycle. Particularly preferred tumor suppressor gene products act on the G1-S transition of the cell cycle. The invention encompasses the use of any tumor suppressor gene product which performs this function, whether known or yet to be discovered. Examples of tumor suppressor genes include p21,

35

p27, p53 (and particularly, the p53175P mutant allele), p57, p15, p16, p18, p19, p73, GADD45 and APC1.

Optionally, one can also use the methods and compositions of the invention to express survival factors in the host cells. Survival factors are intracellular proteins that
5   prevent apoptosis such as bcl-2, bcl-x_L, E1B-19K, mcl-1, crmA, abl, p35, bag-1, A20, LMP-1, Tax, Ras, Rel and NF-κB-like factors. Additionally, all known survival factors, as well as survival factors yet to be discovered, are useful in the methods and compositions of the invention. In yet another embodiment, the tumor suppressor gene(s) is expressed concomitantly with a factor that stabilizes the tumor suppressor gene product in the cell.
10 Examples of stabilizing factors are members of the CAAT enhancer binding protein family. For example, p21 protein activity is stabilized when coexpressed with C/EBPα. Additionally, C/EBPα specifically induces transcription of the endogenous p21 gene. Thus, C/EBPα functions as both a stabilizing factor and as a specific inducer of p21.

Still another aspect of the invention is the use of a nucleotides and methods of the
15 invention to express a gene product that activates cell proliferation. For example, a protein that activates cell proliferation is Mek1, a central protein kinase in the conserved mammalian Ras-MAP signal transduction pathway responding to growth-promoting signals such as cytokines. A particularly preferred version of Mek1 is the Mek1DD mutant (Gruelich and Erikson, 1998, J. Biol. Chem. 273: 13280-13288) described more fully below.
20 Other genetic determinants exerting positive control of mammalian cell cycle that can be used as a protein that activates cell proliferation are cyclins (e.g., cyclin E), Ras, Raf, the MAP kinase family (e.g., MAP, Erk, Sap) E2F, Src, Jak, Jun, Fos, pRB, Mek2, EGF, TGF, PDGF, and a polynucleotide that is antisense to a tumor suppressor gene (e.g., p27 anti-sense expression has been shown to stimulate proliferation of quiescent fibroblasts and
25 enable growth in serum-free medium (Rivard et al.,1996, J. Biol. Chem. 271: 18337-18341.) and nedd5 which is known as positive growth controlling gene (Kinoshita et al., 1997, Genes Dev. 11: 1535-1547).

One aspect of the present invention is illustrated below by the development of a new system for antibiotic-regulated gene expression in eukaryotic cells based on the repressor of
30 a streptogramin resistance operon of S. coelicolor (a Pip). A Pip protein (PIT4), or chimeric Pip proteins (PIT and PIT2) fused to a eukaryotic transactivator were able to control expression of a synthetic eukaryotic promoter (P_{PIR}) containing the P_{ptr}-binding site (in other words, a P_{abr}-linked gene). Genes placed under the control of this PIT/P_{PIR} system were
35 responsive to clinically approved therapeutic compounds belonging to the streptogramin group (pristinamycin, virginiamycin and Synercid) in a variety of mammalian cell lines

(CHO-K1, BHK-21 and HeLa). This novel system exhibited superior inducibility and background expression properties compared to the well-established tetracycline-based system in CHO cells engineered to provide both streptogramin and tetracycline regulation. In these cells, streptogramin does not affect gene expression from the tetracycline-

5 responsive promoter, and therapeutically relevant tetracycline concentrations have only minor effect on expression from the streptogramin-responsive PIT/P_{PIR} system. Therefore, these two different systems can be used together in advanced therapies requiring independent regulation of different transgenes. In addition, responsiveness of the PIT/P_{PIR} system to all type B streptogramins tested indicates that reporter gene expression from P_{PIR}

10 can be used as an efficient high-throughput assay for discovery of new streptogramins. The same concept applies to other eukaryotic antibiotic-responsive transcription regulation systems which can be linked to a reporter gene for the discovery of new antibiotics.

Another aspect of the invention is the development of a double-regulation system particularly advantageous when independent control of at least two different transgenes or

15 sets of transgenes is desired. For example, this aspect of the invention is illustrated by way of a non-limiting example using the combined installation of tetracycline-dependent and pristinamycin-responsive gene regulation in the same cells. In the examples below, a double regulation vector pDuoRex1 was constructed which contains both tetracycline-dependent and pristinamycin-responsive expression units. These units were used, for

20 example, to express the tumor suppressor gene p27 (under the control of the tetracycline-responsive promoter) and a gene product stimulating cell proliferation such as Mek1 (here under the control of P_{PIR}) (pMF195). For advanced gene therapy and tissue engineering strategies, induction of Mek1 (while repressing p27) can activate proliferation of grafted cells and facilitate cell expression while induction of p27 (while repressing Mek1) will

25 arrest cell proliferation which is a desired trait for reimplantation of engineered cells and tissues. Other tumor suppressor genes and other genes stimulating cell proliferation can be substituted.

Still another aspect of the invention are multipurpose expression vectors, as well as cells and methods using the same, which take advantage of the antibiotic dependent

30 activator and repressor systems of the invention. Such vectors can be mono, di- or multicitronic. Non-limiting examples of such vectors are described below by way of working embodiments.

Although the regulated gene expression invention described herein was originally designed for general applications in functional genomic research, gene therapy and tissue

35 engineering, the finding that the streptogramin system illustrated below by way of

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nonlimiting example responds to all commercially available streptogramins, including Synercid, Pyostacin, and virginiamycin, indicates its use as a powerful screening tool for the discovery of novel antibiotics. Accordingly, still another aspect of the invention is a method of screening for candidate antibiotics. The method entails incubating host cells of the

5   invention, the host cells containing a P_{abr} -linked reporter gene and a sequence encoding a P_{abr} -binding protein, in the presence of a test compound, wherein a change in the transcription of the reporter gene indicates that the test compound is a candidate antibiotic.

For example and not by way of limitation, detection of streptogramins is based on addition of metabolic libraries of *Streptomyces* or fungal origin to cultured mammalian cells

10   containing the pristinamycin-responsive reporter system. The presence of streptogramins will downregulate expression of the reporter protein, for example SEAP, driven by P_{PIR}. This screening approach offers two decisive advantages over classical screening technology using indicator bacteria-based antibiogram tests: (i) Antibiotic screening is not limited by the sensitivity of indicator bacteria to a yet uncharacterized streptogramin (sensitivity of

15   bacteria to antibiotics greatly varies between strains and even isolates), and (ii) the mammalian cell-based streptogramin detection concept shows at least one order of magnitude higher sensitivity to this class of antibiotics than antibiogram tests based on bactericidal activity. However, given the suggested interaction of Pip with other classes of antibiotics (a list is given in Salah-Bey K, Blanc, V., and Thompson C.J, 1995; Mol.

20   Microbiol. 17, 1001-1012) the Pip/P_{PIR}-based mammalian gene regulation system can detect other antibiotic compounds. Also, extension of this detection concept to include antibiotic-responsive reporter gene expression using other AIP and P_{abr} components from other Actinomycetes is within the scope of this invention.

The invention having been described, the following examples are offered by way of

25   illustration and not limitation.

Example 1: Steptogramin-repressible Mammalian Gene Regulation System

After showing that the presence of PI reverses Pip association with P_{PTR} *in vitro*, this example describes the use of Pip and P_{PTR} to design a novel streptogramin-responsive

30   mammalian gene regulation system with excellent regulatory properties which is functionally compatible with the tetracycline-based transcription regulation system (TET system) most widely used presently.

35

METHODS

Construction of the streptogramin-dependent transactivator (PIT) and the pristinamycin-regulatable promoter P$_{PIR}$

PIT, the fusion protein of Pip and the VP16 C-terminal transactivation domain of

5   *Herpes simplex* (Triezenberg *et al.*, 1988, Genes Dev. 2, 718-729) was constructed by amplifying the *S. coelicolor* Pip from pGemT::Epip4 with oligos:

OMF63: GTACGAATTCCCACCATGAGTCGAGGAGAGGTG (SEQ ID NO:1) and

OMF64: GCGCGCGGCTGTACGCGGAGGCCTGTTCGACCATC (SEQ ID NO:2) followed by cloning into vector pcDNA3.1/V5/His-TOPO (Invitrogen) under control of P$_{CMV}$(pMF150).

10 These primers can also be used to clone the desired gene sequences from *S. pristinaespiralis* genomic DNA.

P$_{PIR}$ seqence (SEQ ID NO:3):

GATCGACGTCGGAGAAATAGCGCTGTACAGCGTATGGGAATCTCTTGTACGGTGTACGAGT
ATCTTCCCGTACACCGTACAAGGAGCCTGCAGGgagtaccctcgaccgccgGAGTATAAAT

15 AGAGGCGCTTCGTCTACGGAGCGACAATTCAATTCAAACAAGCAAAGTGAACACGTCGCTA
AGCGAAAGCTAAGCAAATAAACAAGCGCAGCTGAACAAGCTAAACAATCTGCAGTAAAGTG
CAAGTTAAAGTGAATCAATTAAAAGTAACCAGCAACCAAGTAAATCAACTGCAACTACTGA
AATCTGCCAAGAAGTAATTATTGAATACAAGAAGAGAACTCTGAATACTTTCAACAAGTTA
CCGAGAAAGAAGAACTCACACACAGCTAGCGTTTAAACTTAAGCTTGGTACCGAGCTCGGA
TCCACTAGTCCAGTGTGGTGGAATTC.

20   The Pip-containing *SspI*/*BssH*II fragment of pMF150 was subsequently cloned into the corresponding sites of pSBC2-tTA (pSAM200 (*SspI*/*BssH*II) (Fussenegger *et al.*, 1997, Biotechnol. Prog. 13, 733-740) thereby replacing the TetR Domain of tTA by Pip. The resulting plasmid has the following PIT coding sequence:

PIT sequence (SEQ ID NO:4):

25 ATGAGTCGAGGAGAGGTGCGCATGGCGAAGGCAGGGCGGGAGGGGCCGCGGGACAGCGTGT
GGCTGTCGGGGGAGGGGCGGCGCGGCGGTCGCCGTGGGGGGCAGCCGTCCGGGCTCGACCG
GGACCGGATCACCGGGGTCACCGTCCGGCTGCTGGACACGGAGGGCCTGACGGGGTTCTCG
ATGCGCCGCCTGGCCGCCGAGCTGAACGTCACCGCGATGTCCGTGTACTGGTACGTCGACA
CCAAGGACCAGTTGCTCGAGCTCGCCCTGGACGCCGTCTTCGGCGAGCTGCGCCACCCGGA
CCCGGACGCCGGGCTCGACTGGCGCGAGGAACTGCGGGCCCTGGCCCGGGAGAACCGGGCG
30 CTGCTGGTGCGCCACCCCTGGTCGTCCCGGCTGGTCGGCACCTACCTCAACATCGGCCCGC
ACTCGCTGGCCTTCTCCCGCGCGGTGCAGAACGTCGTGCGCCGCAGCGGGCTGCCCGCGCA
CCGCCTGACCGGCGCCATCTCGGCCGTCTTCCAGTTCGTCTACGGCTACGGCACCATCGAG
GGCCGCTTCCTCGCCCGGGTGGCGGACACCGGGCTGAGTCCGGAGGAGTACTTCCAGGACT
CGATGACCGCGGTGACCGAGGTGCCGGACACCGCGGGCGTCATCGAGGACGCGCAGGACAT
CATGGCGGCCCGGGGCGGCGACACCGTGGCGGAGATGCTGGACCGGGACTTCGAGTTCGCC
35 CTCGACCTGCTCGTCGCGGGCATCGACGCGATGGTCGAACAGGCCTCCGCGTACAGCCGCG
CGCGTACGAAAAACAATTACGGGTCTACCATCGAGGGCCTGCTCGATCTCCCGGACGACGA

CGCCCCCGAAGAGGCGGGGCTGGCGGCTCCGCGCCTGTCCTTTCTCCCCGCGGGACACACG
CGCAGACTGTCGACGGCCCCCCCGACCGATGTCAGCCTGGGGGACGAGCTCCACTTAGACG
GCGAGGACGTGGCGATGGCGCATGCCGACGCGCTAGACGATTTCGATCTGGACATGTTGGG
GGACGGGGATTCCCCGGGTCCGGGATTTACCCCCCACGACTCCGCCCCCTACGGCGCTCTG
GATATGGCCGACTTCGAGTTTGAGCAGATGTTTACCGATGCCCTTGGAATTGACGAGTACG

5   GTGGGTAG.

PIT2, the fusion protein of Pip and the p65 transactivation domain of the human NF-κB transcription activator was cloned in a three step procedure: (i) The p65 domain of the human NF-κB transcription activator was amplified from pSG424-p65 (kindly provided by H. Hauser, GBF, Braunschweig, Germany) with oligos OMF97:

10 GATCGCGCGCATGATGAGTTTCCCACC (SEQ ID NO:22) and OMF98:

GATCAAGCTTGGATCCTTAGGAGCTGATCTGACT (SEQ ID NO:23) and cloned in sense orientation into pcDNA3.1/V5/His-TOPO (Invitrogen) to result in plasmid pMF197. (ii) pMF197 was restricted with BssHII and HindIII contained in the oligos (underlined) and the p65 domain was ligated to the corresponding sites (BssHII/HindIII) of pSBC2-tTA

15 (Fussenegger et al., 1997) thereby replacing the VP16 domain of the tetracycline-dependent transactivator and resulting in plasmid pMF204. (iii) The tetR domain was excised from pMF204 by SspI/BssHII and replaced by Pip excised from pMF150 by SspI/BssHII to give plasmid pMF206. pMF206 contains the following PIT2 coding sequence:

ATGAGTCGAGGAGAGGTGCGCATGGCGAAGGCAGGGCGGGAGGGGCCGCGGGACAGCGTGT

20 GGCTGTCGGGGGAGGGGCGGCGCGGCGGTCGCCGTGGGGGGCAGCCGTCCGGGCTCGACCG
GGACCGGATCACCGGGGTCACCGTCCGGCTGCTGGACACGGAGGGCCTGACGGGGTTCTCG
ATGCGCCGCCTGGCCGCCGAGCTGAACGTCACCGCGATGTCCGTGTACTGGTACGTCGACA
CCAAGGACCAGTTGCTCGAGCTCGCCCTGGACGCCGTCTTCGGCGAGCTGCGCCACCCGGA
CCCGGACGCCGGGCTCGACTGGCGCGAGGAACTGCGGGCCCTGGCCCGGGAGAACCGGGCG
CTGCTGGTGCGCCACCCCTGGTCGTCCCGGCTGGTCGGCACCTACCTCAACATCGGCCCGC

25 ACTCGCTGGCCTTCTCCCGCGCGGTGCAGAACGTCGTGCGCCGCAGCGGGCTGCCCGCGCA
CCGCCTGACCGGCGCCATCTCGGCCGTCTTCCAGTTCGTCTACGGCTACGGCACCATCGAG
GGCCGCTTCCTCGCCCGGGTGGCGGACACCGGGCTGAGTCCGGAGGAGTACTTCCAGGACT
CGATGACCGCGGTGACCGAGGTGCCGGACACCGCGGGCGTCATCGAGGACGCGCAGGACAT
CATGGCGGCCCGGGGCGGCGACACCGTGGCGGAGATGCTGGACCGGGACTTCGAGTTCGCC
CTCGACCTGCTCGTCGCGGGCATCGACGCGATGGTCGAACAGGCCTCCGCGTACAGCCGCG

30 CGCATGATGAGTTTCCCACCATGGTGTTTCCTTCTGGGCAGATCAGCCAGGCCTCGGCCTT
GGCCCCGGCCCCTCCCCAAGTCCTGCCCCAGGCTCCAGCCCCTGCCCCTGCTCCAGCCATG
GTATCAGCTCTGGCCCAGGCCCCAGCCCCTGTCCCAGTCCTAGCCCCAGGCCCTCCTCAGG
CTGTGGCCCCACCTGCCCCCAAGCCCACCCAGGCTGGGGAAGGAACGCTGTCAGAGGCCCT
GCTGCAGCTGCAGTTTGATGATGAAGACCTGGGGGCCTTGCTTGGCAACAGCACAGACCCA
GCTGTGTTCACAGACCTGGCATCCGTCGACAACTCCGAGTTTCAGCAGCTGCTGAACCAGG

35 GCATACCTGTGGCCCCCCACACAACTGAGCCCATGCTGATGGAGTACCCTGAGGCTATAAC

TCGCCTAGTGACAGGGGCCCAGAGGCCCCCCGACCCAGCTCCTGCTCCACTGGGGGCCCCG
GGGCTCCCCAATGGCCTCCTTTCAGGAGATGAAGACTTCTCCTCCATTGCGGACATGGACT
TCTCAGCCCTGCTGAGTCAGATCAGCTCCTAA (SEQ ID NO:24).

For construction of P_{PIR} the minimal promoter of the *Drosophila* heat-shock gene

5   *hsp70* $P_{hsp70min}$; bp -40 to 241; Corces *et al.*, 1984, J. Biol. Chem. 259, 14812-14817)
contained on pTRIDENT7 (Fussenegger *et al.*, 1998, Biotechnol. Bioeng. 57, 1-10) was
amplified by PCR using oligos
OMF62:CTTGTACGGTGTACGAGTATCTTCCCGTACACCGTACAAGGAGCCTGCAGGgagt
accctcgaccgccg (SEQ ID NO:5) and OMF57: GATCCATCGATTGATCAGGCGC (SEQ

10   ID NO:6) and cloned in anti-*lacZ* orientation into the pCR2.1-TOPO vector (Invitrogen) to
give plasmid pMF153. The premature P_{PIR} sequence contained in pMF153 was subjected to
a second round of PCR using primers OMF69:
GATCGACGTCGGAGAAATAGCGCTGTACAGCGTATGGGAATCTcttgtacggtgtacgagt
atc (SEQ ID NO:7) and OMF57 described above. The resulting PCR fragment containing

15   the complete P_{PIR} sequence was cloned into pCR2.1-TOPO in *lacZ* orientation to give
pMF161. The annealing sequence of OMF69 (small letters) is complementary to the
extension of OMF62 (capital letters) and adds the remaining Pip binding site of P_{PTR} to P_{PIR}.
*Construction of the pristinamycin-responsive reporter plasmids pMF164 and pMF172 and
the pTWIN vectors*

20   For the construction of pMF164 the P_{PIR}-containing fragment was excised from
pMF161 by *Aat*II and *Eco*RI and ligated to the corresponding sites (*Aat*II/*Eco*RI) of the
GFP expression vector pMF104, thereby replacing the TET-responsive promoter $P_{hCMV*-1}$
(Fussenegger *et al.*, 1998, *supra*). Similarly, pMF172 was constructed for quantitative
analysis of P_{PIR}-mediated expression, by exchanging $P_{hCMV*-1}$ of pMF111 (Fussenegger *et al.*,

25   1997, Biotechnol. Bioeng. 55, 927-939) by P_{PIR} of pMF164 via *Ssp*I and *Eco*RI.

pTWIN contains both PIT and tTA in a P_{SV40}-driven dicistronic configuration. For
construction of pTWIN, PIT was first transferred from pMF156 as an *Eco*RI/*Hind*III
fragment to the corresponding sites of pSBC-1 (*Eco*RI/*Hind*III; resulting in pMF167) before
the PIT expression unit could be fused to the tTA expression unit contained on pSBC2-tTA

30   (Fussenegger *et al.*, 1997, *supra*) via *Ssp*I/*Not*I sites. Thus, pTWIN contains the dicistronic
P_{SV40}-PIT-IRES-tTA-pA expression cassette.
*Cell culture, transfection, construction of stable cell lines, SEAP activity test and gel
retardation assays*

Chinese hamster ovary cells (CHO-K1, ATCC: CCL 61), Baby hamster kidney cells

35   (BHK-21, ATCC: CRL-8544), and HeLa cells (ATCC: CRL-7923) as well as stable cell

lines CHO-PIT1, CHO-PIT2, CHO-TWIN1$_{95}$, CHO-TWIN1$_{108}$ and CHO-PIT1-SEAP were cultured as described before (Fussenegger *et al.*, 1998, Nat. Biotechnol. 16,468-472) in the presence of appropriate antibiotics: G418 400 μg/ml; zeocin 100 μg/ml. Optimized CaPO$_4$ protocols were used for high efficiency transient transfection of all cell lines (*id*). Transient

5   transfected cells were routinely analyzed after 48 h for GFP or SEAP expression using fluorescence microscopy and p-nitrophenolphosphate-based light absorbance timecourse, respectively, as described before (Fussenegger *et al.*, 1998, Nat. Biotechnol. 16, 468-472; Berger *et al.*, 1988, Gene 66, 1-10 b). For construction of stable CHO-PIT and CHO-TWIN cell lines, CHO-K1 was cotransfected either with pMF156 or pTWIN1 and pSV2*neo*

10 carrying the G418 resistance gene. CHO-PIT1-SEAP was constructed by cotransfection of pMF172 and pZeoSV2 (Invitrogen). For assessment of dose-response characteristics of P$_{PIR}$-regulated gene expression, CHO-PIT1-SEAP were cultured at cell densities of 150,000/ml for 48 at various PI concentrations. The mixed populations were cloned using FACS-mediated single-cell-sorting (FACStar^{Plus}; Beckton Dickinson). Gel retardation assay

15 were performed as described (Salah-Bey *et al.*, 1995, *supra*) using Pip purified from an overproducing strain of *E. coli*. Pip (0.02pmol) was titrated with a range of antibiotic concentrations in a reaction volume of 20 μl. Protein solutions were pre-incubated at room temperature for 15 min. in the absence or presence of PI or its derivative quinupristin in a buffer containing 10 mM Tris (pH 7.8), 10 mM MgCl$_2$, 300mM Nacl, 2mM DTT and 10%

20 glycerol. An *EcoRI-HindII* P$_{PTR}$ fragment (0.02pmol) containing three Pip binding sites (radiolabelled using α-^{32}P)dATP was added and incubated for 15 min. To minimize non-specific protein binding and increase protein stability, 2μg of poly dIdC and 20μg BSA were included in the incubation mix. Protein-DNA complexes were separated on 5% polyacrylamide, TBE gels and visualized by autoradiography.

25 *Regulating streptogramins*

Five different sources of pristinamycin were used: The two therapeutic forms Pyostacin™ and Synercid™, pristinamycin discs, and the single compounds pristinamycin I (PI) and pristinamycin II (PII). Pyostacin™ pills (500 mg) were ground in a mortar and solubilized in DMSO or water at a stock concentration of 50 mg/ml. Synercid™ (RP

30 59500; Lot Nr. CB06253) was provided by Aventis in injection-ready vials containing 500 mg lyophilized antibiotic which was reconstituted in 5 ml of 5% glucose solution and frozen at -20°C. Individual pristinamycin compounds PI and PII (identical to virginiamycin M$_1$ (VM$_1$); Sigma ref. V2753) were solubilized in DMSO at a concentration of 50 mg/ml and

35 kept at -20°C. Antibiotic discs containing 15 μg pristinamycin or virginiamycin (bioMerieux, ref. 54552; bioMerieux, ref. 54612) were soaked in 1 ml cell culture medium

at 4¡C for up to 24 h. Virginiamycin consists of virginiamycin S_1 (VS_1), the PI analogue, and virginiamycin M_1, which is identical in structure to PII (Barri re *et al.*, 1994, *supra*). 2 µg/ml of streptogramin B was routinely used for regulation studies in cell culture. The concentration of individual streptogramin components was calculated based on their fixed

5   70:30 ratio (w/w; PII/VM_1/dalfopristin : PI/VS_1/quinupristin). For control experiments TET and doxycycline (DOX) were used either as discs (30 µg/disc, bioMⒹrieux ref. 54882 (TET) and ref. 54172 (DOX)) or as powder (Sigma refs. T3383 and D9891).

RESULTS

Construction and functional studies of the streptogramin-based gene regulation system in

10   *CHO cells*

Gel retardation assays using purified Pip protein and a DNA fragment containing P_{PTR} target sequence indicated a strong *in vitro* interaction between both elements in the absence of pristinamycin I. However, in the presence of PI, Pip was completely released from P_{PTR} (Fig. 1). P_{PTR} of *S. pristinaespiralis* contains 3 dyad symmetrical binding sites

15   (Fig. 1; lane A; P_{PTR} in the absence of Pip) all of which are saturated at relatively large amounts of purified Pip (0.4 pmol; lane A) resulting in a single shifted band. Smaller amounts of Pip (0.02 pmol) presented an intermediate situation in which all possible permutations of site occupancy are represented in three distinct retarded bands (lane C). Pip (0.2 pmol lanes D-M) was titrated with a range of PI from 50 pmol to 0.01 pmol (lanes D-H;

20   D: 50 pmol; E: 10 pmol; F: 1 pmol; G: 0.1 pmol; H: 0.01 pmol). Pip was completely released from P_{PTR} by excess molar concentrations of PI. Under identical conditions the semisynthetic PI derivative quinupristin shows only partial release of Pip from individual P_{PTR} binding sites even at highest concentration (lanes I-M; I: 50 pmol; J: 10 pmol; K: 1 pmol; L: 0.1 pmol; M: 0.01 pmol). The relatively low Pip-releasing activity of quinupristin

25   correlates with its lower efficiency in regulating the PIT/P_{PIR} system (see Table 5, *infra*).

In order to analyze the potential of the Pip/P_{PIR} system for the design of a novel mammalian gene regulation system, we adapted these *Streptomyces* regulatory elements for use in a eukaryotic context by construction of a set of two chimeric determinants: the PI-inhibited transactivator (PIT, Pip fused to the VP16 transactivation domain of the *Herpes*

30   *simplex* virus; Triezenberg *et al.*, 1988, *supra*) and the PI-responsive promoter (P_{PIR}) (P_{PTR} fused to the minimal insect promoter $P_{hsp70min}$; Corces *et al.*, 1984, *supra*). Following transfection of a P_{PIR}-driven GFP expression construct (pMF164) into CHO cells, no green fluorescence could be observed by fluorescence microscopy, indicating that no endogenous host factors activate P_{PIR}. GFP-expression could only be detected when pMF164 was

35   cotransfected with PIT-encoding pMF156, showing that the chimeric PIT protein functions

as a transactivator for P_{PIR} in mammalian cells. Transactivation of GFP expression was strictly PIT-dependent and could not be achieved by cotransfection of pMF164 with vectors containing other transactivators such as the tetracycline- (TET-) or ecdysone-dependent transactivators (pUHD15-1 encoding the tetracycline-repressible transactivator tTA;

5   pUHD17-1*neo* encoding the tetraycline-inducible reverse tTA (rtTA; Gossen *et al.*, 1995, *supra*; pVgRXR encoding VgEcR and RXR which heterodimerize to form the ecdysone-dependent transactivator; No *et al.*, 1996, *supra*). Also PIT did not activate GFP expression from TET- or ecdysone-dependent promoters (pMF104; pIND-GFP (Invitrogen)).

Addition of pristinamycin to the culture medium of CHO cells simultaneously

10   transfected with PIT (pMF156) and a P_{PIR}-SEAP (human placental secreted alkaline phosphatase) reporter construct (pMF172) greatly decreases SEAP expression compared to a cotransfected culture without pristinamycin as shown in Table 2.

Inducer	SEAP Production (%)
None	100
Pristinamycin (Pyostacin®; 2 μg/ml)	8.49 ± 0.7
PI (2 μg/ml)	7.90 ± 1.2
PII (2 μg/ml)	96.3 ± 3.2

Table 2: Regulation potential of pristinamycin (Pyostacin®) and the pristinamycin group B (PI) and group A (PII) compounds. CHO-K1 cells were simultaneously transfected with pMF156 (PIT) and pMF172 (P_{PIR}-SEAP) in the presence or absence of the inducer indicated. Reporter gene expression (SEAP) was assayed 48 hr later.

In addition, pMF206 (PIT2; Pip-p65) was cotransfected with pMF172 (P_{PIR}-SEAP) and the SEAP readout in the absence and presence of PI (2 μg/ml) was directly compared to

30   CHO-K1 cells cotransfected with pMF156 (PIT; Pip-VP16) and pMF172 (P_{PIR}-SEAP). As shown in the following table, PIT2 mediates higher overall SEAP expression levels compared to the pMF156/pMF172 configuration in the absence of pristinamycin but basal expression levels in the presence of pristinamycin remain significantly higher.

35

	No Inducer	+PI (2µg/ml)
CHO + pMF206 + pMF172	0.49 ± 0.04	0.31 ± 0.003
CHO + pMF156 + pMF172	0.23 ± 0.007	0.03 ± 0.002

Table 3 (previous page): Comparison of the regulation characteristics of PIT and PIT2. CHO-K1 cells were simultaneously cotransfected with the plasmids indicated and the SEAP readout (slope of the p-nitrophenolphosphate-based light absorbance time courses) was determined in the presence and absence of pristinamycin 48 hr post transfection.

This experiment indicates that external pristinamycin can enter mammalian cells and exert control of PIT/P_{PIR}-regulated gene expression there. However, pristinamycin has no influence on expression levels of P_{SV40} or P_{CMV}-driven SEAP expression constructs (data not shown). Interestingly, only addition of the group B streptogramin PI shows effective downregulation of PIT/P_{PIR}-mediated SEAP expression; PII did not display any significant repression activity. Similar PI-dependent gene expression using the PIT/P_{PIR} system has also been observed in BHK-21 and HeLa cells (not shown). No deleterious effects on CHO cell morphology and growth were observed at PI concentrations of 2 µg/ml found to be effective for repression of the PIT/P_{PIR} system. This is the same order of magnitude as tissue concentrations reached in antibiotic therapy. Only much higher PI concentrations (above 50 µg/ml) were toxic for CHO cells.

Stable expression of PIT in CHO cells

Two representative clones, CHO-PIT1 and CHO-PIT2, were chosen at random among 11 PIT-expressing CHO cell clones stably transfected with a constitutive PIT expression construct (pMF156). Both cell lines show no unusual cell morphologies and display similar growth behavior compared to wild-type CHO-K1 cells, indicating that sustained constitutive PIT expression does not have obvious deleterious physiological effects on CHO cells.

Transient transfection of CHO-PIT1 and CHO-PIT2 with a P_{PIR}-SEAP expression vector (pMF172) resulted in high level SEAP expression in the absence of PI and significant repression in the presence of 2 µg/ml PI as shown in Table 4.

	SEAP Production (%)	
	-PI	+PI
CHO-PIT1 + P_{PIR}-SEAP (pMF172)	100	7.50 ± 2.32
CHO-PIT2 + P_{PIR}-SEAP (pMF172)	100	12.7 ± 3.57

Table 4: Pristinamycin I- (PI) dependent SEAP production of PIT- (PI-dependent transactivator) expressing stable cell lines CHO-PIT1 and CHO-PIT2 transiently transfected with the SEAP-encoding, PI-responsive reporter plasmid pMF172.

Induction factors (the ratio of SEAP activity without PI to SEAP activity with PI) reach 13 and 8 for CHO-PIT1 and CHO-PIT2, respectively. PI-responsive SEAP regulation of both CHO-PIT derivatives is fully reversible following repeated cycles of addition and withdrawal of this streptogramin, indicating reversible PI-PIT interaction in mammalian cells. This characteristic is necessary to achieve fluctuating daily dosing regimes optimal for many therapeutic proteins such as insulin.

Dose-dependence of PI-mediated gene regulation in CHO cells

The levels of P_{PIR}-regulated gene expression could be controlled by varying the concentrations of PI used for induction. This was assessed using a stable CHO cell line (CHO-PIT1-SEAP) which expresses P_{PIR}-SEAP (pMF172) and contains also a constitutive PIT expression vector (pMF156). Fig. 2 shows the dose-response curve for PI-dependent SEAP expression of CHO-PIT1-SEAP cultures over 5 orders of magnitude of PI concentration. Beyond the concentration window of 10-500 ng/ml, the PIT/P_{PIR} is either fully induced (< 10 ng/ml) or repressed (> 500 ng/ml), while gene expression can be adjusted to different levels within this window of concentrations. Increasing PI concentration above 10 ng/ml leads to a gradual decrease in SEAP expression, with lowest SEAP expression levels at PI concentrations over 500 ng/ml. Besides the response plateaus of the PIT/P_{PIR} system at PI concentrations below 10 ng/ml and above 500 ng/ml, this system shows a broad window between these two concentrations in which the gene expression can be adjusted to intermediate levels. Such adjustable gene expression characteristics are particularly important for clinical applications which require titration of circulating proteins into the therapeutic range.

Regulation efficiency of different streptogramins

Access to alternative effective streptogramins would expand the spectrum of regulating agents available for future use in human cell and gene therapy. Accordingly, several commercially available streptogramin sources were tested for their potential to
5   regulate the PIT/P_{PIR} system as shown below in Table 5. Among available streptogramins, virginiamycin proved to be the most efficient regulating agent, showing an induction factor of 32, almost three times higher than pristinamycin-based regulation.

	SEAP Production (%)
No streptogramin	100
Pristinamycin I	8.06 ± 0.05
Virginiamycin	3.41 ± 0.47
Synercid	28.8 ± 3.73

Table 5: Regulation potential of different streptogramins. CHO-PIT1 was transiently transfected with the SEAP-encoding plasmid pMF172 and grown for 48 h in the absence or presence of different streptogramins at a concentration of 2 μg/ml of their group B component.

25   Virginiamycin is very similar in structure to pristinamycin, since its group A component virginiamycin M_1 is identical to PII and its group B constituent, virginiamycin S_1 (VS_1), differs from PI only by the lack of a dimethylamine group.

The improved antibiotic therapeutic potential of Synercid resides in its water solubility. Although Synercid was expected to achieve better control of the PIT/P_{PIR} system
30   due to its greater water solubility, its repression activity was almost 4-fold lower compared to Pyostacin and PI (Table 5). Apparently, the [(5δR)-[(3S)-quinuclidinyl]-thiomethyl modification of PI decreased the affinity of quinupristin to PIT, which is supported by gel retardation assays showing that quinupristin releases Pip from its target sequence P_{PTR} less efficiently than PI (Fig. 3).
35

Comparison and compatibility studies of the streptogramin- and tetracycline-based gene regulation systems in CHO cells

The regulation performance of the PI-based regulation system was compared directly with the widely used TET-responsive expression concept. pTWIN1 encoding both PIT and

5   the tetracycline-dependent transactivator tTA in a single constitutive dicistronic expression unit was stably established in CHO-K1 cells. Two representative clones out of 20 pTWIN1-containing clones, CHO-TWIN1$_{95}$ and CHO-TWIN1$_{108}$, were chosen for further studies. CHO-TWIN cells show the same cell morphology and similar growth behavior as wild-type CHO cells.

10   CHO-TWIN1$_{95}$ and CHO-TWIN1$_{108}$ were transfected under identical conditions with the isogenic plasmids containing the SEAP gene under control of the tTA-activated promoter $P_{hCMV*-1}$ (pMF111) or the PIT-activated promoter P_{PIR} (pMF172). Transfected cells were grown for 48 h in the presence or absence of either antibiotic prior to assay of SEAP activity. Results are shown below in Table 6.

15

		SEAP Production (%)	
		CHO-TWIN1$_{95}$	CHO-TWIN1$_{108}$
P_{PIR}-SEAP (pMF172)	No inducer	100	100
	+ PI	8.84 ± 0.01	2.2 ± 0.4
	+VIR	3.75 ± 0.39	1.2 ± 0.41
$P_{hCVM*-1}$-SEAP (pMF111)	No inducer	100	100
	+ TET	8.57 ± 0.37	7.56 ± 0.01

Table 6: Comparison of the regulation performance of streptogramin- (PI and virginiamycin; VIR) and tetracycline-based gene regulation systems. The two cell clones CHO-TWIN1$_{95}$ and CHO-TWIN1$_{108}$ containing both pristinamycin- and tetracycline-dependent transactivators were transfected with either the streptogramin-responsive or the

35   TET-responsive SEAP reporter plasmids pMF172 (P_{PIR}-SEAP) and pMF111 ($P_{hCMV*-1}$-

SEAP), respectively, and the relative SEAP production was assessed. SEAP production
values are normalized by SEAP activities under the antibiotic-free, active promoter
conditions as follows: pMF172 in CHO-TWIN1$_{95}$: 1.7mU/ml; in CHO-TWIN1$_{108}$:
1.8mU/ml. pMF111 in CHO-TWIN1$_{95}$: 18.6mU/ml; in CHO-TWIN1$_{108}$: 19.2mU/ml.

5   The maximum SEAP expression levels of the TET-system were approximately 10-
fold higher compared to the P$_{PIR}$-driven SEAP expression. This is expected since the
different chimeric promoters involved differ in their minimal promoter elements. While the
TET-promoter P$_{hCMV*-1}$ has been optimized by heptameric tTA binding elements to reach 35-
fold higher expression levels than the precursor viral CMV promoter (Yin *et al.*, 1995,
10   Cancer Res. 55, 4922-4928), P$_{PIR}$ is in its unmodified, original "wild-type" configuration
prior to any such refinements to increase fully-activated transcription. To analyze the
relative regulatory characteristics, we evaluated induction factors (IF; ratio of maximal
expression level to antibiotic-repressed expression level). IFs have been shown to remain
largely unaffected by the choice of the minimal promoter or other promoter modifications
15   aimed at increasing the maximum expression level (No *et al.*, 1996, *supra*). While the PI-
and the TET-system of CHO-TWIN1$_{95}$ exhibit nearly identical IFs of 12 in the SEAP
reporter assay, CHO-TWIN1$_{108}$ displays 13-fold downregulation of SEAP in the presence of
TET but downregulation by a factor of 45 by the addition of PI. Using virginamycin as
regulating agent instead of PI for CHO-TWIN1$_{95}$ and CHO-TWIN1$_{108}$ transfected with the
20   P$_{PIR}$-SEAP expression vector (pMF172) IFs are even higher (27 for CHO-TWIN1$_{95}$ and 83
for CHO-TWIN1$_{108}$) (Table 6). Therefore, the PIT/P$_{PIR}$-based mammalian gene regulation
system shows superior gene inducibility characteristics than the TET system in CHO cells
engineered to provide regulation of both systems.

        The PI and the TET systems can be used for combined therapeutic applications
25   requiring different control modalities for different transgenes since they are compatible and
regulated by different therapeutic antibiotics for which large sets of clinical and
pharmacokinetic data are available. Crossrepression of PI and TET (or the TET derivative
doxycycline) on the other expression regulation system was evaluated for CHO-TWIN1$_{108}$
transfected separately with P$_{PIR}$-SEAP (pMF172) and P$_{hCMV*-1}$-SEAP (pMF111) expression
30   constructs, and the results shown in Table 7.

35

		SEAP Production (%)
CHO-TWIN1$_{108}$ + pMF111	-	100
CHO-TWIN1$_{108}$ + pMF111	+ PI (2 µg/ml)	94.8 ± 5.3
CHO-TWIN1$_{108}$ + pMF111	+ PI (10 µg/ml)	92.4 ± 7.9
CHO-TWIN1$_{108}$ + pMF172	-	100
CHO-TWIN1$_{108}$ + pMF172	+ TET (2 µg/ml)	87.7 ± 1.76
CHO-TWIN1$_{108}$ + pMF172	+ TET (10 µg/ml)	57.5 ± 5
CHO-TWIN1$_{108}$ + pMF172	+ DOX (2 µg/ml)	77 ± 6.9
CHO-TWIN1$_{108}$ + pMF172	+ DOX (10 µg/ml)	50.5 ± 7.9

Table 7: Compatibility of the streptogramin- and tetracycline-based gene regulation systems. Crossregulation of pristinamycin (PI) and the tetracyclines doxycycline (DOX) and tetracycline (TET). The cell line CHO-TWIN1$_{108}$ which contains the tetracycline-dependent transactivator (tTA) as well as the pristinamycin-dependent transactivator (PIT) was transfected with the streptogramin-responsive reporter SEAP plasmid pMF172 (P$_{PIR}$-SEAP) or the tetracycline-responsive SEAP reporter plasmid pMF111 (P$_{hCMV*-1}$-SEAP).

PI shows no effect on expression from the TET-responsive system. This experiment indicates that PI activation of SEAP expression in constructs containing PIT and P$_{PIR}$ operates by specific interaction of PI and PIT, as occurs between PI and the *S. coelicolor* protein Pip (Table 7; Fig. 5), and not via any generalized, nonspecific effect of PI on CHO cells.

Both tetracycline derivatives downregulate the PIT/P$_{PIR}$ system in a dose-dependent manner. However, at tetracycline concentrations commonly used for regulation of the TET-

responsive promoter (2 µg/ml), the repressive effect of TET on the PIT/P_{PIR} system is only 17%. This is sufficiently low that, for practical purposes, the TET- and Pip/P_{PIR} systems provide essentially independently regulatable expression.

<div align="center">DISCUSSION</div>

5   Properties of an ideal gene regulation system for therapeutic use include (i) low baseline expression and high induction ratio, (ii) control by a readily bioavailable, small-molecule drug showing no interference with host metabolism, (iii) high pharmacokinetic turnover of the regulating agent in all tissues to allow rapid reversion to the wild-type configuration, (iv) low potential for immune recognition, and (v) high modularity of

10   regulation components to allow independent and efficient optimization as well as adaptation of the system to specific therapeutic situations. The streptogramin-based mammalian gene regulation system described here ideally satisfies these requirements. In cell lines engineered to afford control of both systems, the streptogramin-based regulation system shows over 10-fold lower baseline expression levels and up to 4-fold higher induction

15   rations than the TET-responsive system. More importantly, streptogramins and tetracycline regulate their respective mammalian transcription systems essentially independently, showing that these two systems can be used together to achieve advanced therapeutic regimens in which to sets of transgenes can be regulated separately.

         A whole set of streptogramin antibiotics, including the recently approved Synercid˙,

20   are available with confirmed excellent bioavailability, pharmacokinetics and human compatibility. Also, streptogramin antibiotics are actively taken up by human cells, leading to regulation-effective concentrations in almost every part of the human body (Bebear, *et al.*, 1997, J. Antimicob. Chemother. 39, 59-62). Nevertheless, streptogramin antibiotics are rapidly eliminated from the blood and most tissues, with half-lives typically not exceeding

25   one hour in humans (Bergeron *et al.*, 1997, J. Antimicrob. Chemother. 39, 129-138), ensuring ready reversibility of possible therapeutic conditions. The brain is the only site where virtually no streptogramin could be found following oral or intravenous application, since this class of antibiotics is unable to cross the blood-brain barrier (Bergeron *et al.*, 1997, *supra*). This natural tissue-specific selectivity could allow straightforward brain-

30   exclusive expression of the PIT/P_{PIR} system in the presence of regulation-effective concentrations of streptogramin in the remaining body parts to enable design of therapeutic strategies for neurodegenerative diseases (Hardy *et al.*, 1998, Science 282, 1075-1079).

         A mammalian gene regulation system such as the PIT/P_{PIR} configuration which responds to a class of chemically related antibiotics offers another powerful new dimension:

35   finding new antibiotics which are urgently needed to cope with increasing prevalence of

<div align="center">- 31 -</div>

multidrug resistant bacterial pathogens. Metabolic libraries from *Streptomyces* or fungi could be screened using cultured mammalian cells containing the PIT/P$_{PIR}$ system linked to a reporter gene to identify novel streptogramins. This technology is at least one order of magnitude more sensitive than classical microbial inhibition tests and not biased by

5   antibiotic resistance of indicator bacteria.

Additionally, the system described above can be adapted to use any of a number of different antibiotic resistance systems found in secondary metabolite/antibiotic producers within the *Actinomycetes*, particularly the *Streptomyces*. Examples of such systems that contain P$_{ptr}$- responsive promoters with cognate antibiotic binding proteins are the

10   rifamycin-responsive gene cluster, the daunorubicin-responsive gene cluster, the landomycin-responsive gene cluster, the rapamycin-responsive gene cluster, and the tetracenomycin-responsive gene cluster.

Example 2: The Double Regulation System

15   Most of today's gene therapy and tissue engineering strategies focus on stable integration of transgenes into human somatic cells either *in vivo* or *ex vivo*. While initial success was achieved using sophisticated gene transfer technology including attenuated viruses, site-specific recombination for targeted integration and non-immunogenic selection markers, gene transfer is not the only challenge in future gene therapy and tissue

20   engineering. However, the success and realization of this technology will largely dependent on flanking concepts which allow *ex vivo* expansion of grafted tissue followed by sustained growth control and reimplantation of treated cells or tissues. This concept requires two consecutive steps of opposite proliferation control which enables first expression of genes which activate proliferation for *ex vivo* expansion of tissue cells followed then by gene

25   therapeutic operation and activation of proliferation control to allow reimplantation of genetically engineered tissue.

Using two human-compatible gene regulation systems, the tetracycline- and the pristinamycin-responsive system we set out to construct a double regulation system to achieve completely externally controlled proliferation management of mammalian cells.

30   MATERIALS AND METHODS

In order to construct the double regulatable mammalian expression vector pDuoRex1 a P$_{PIR}$-pA containing fragment was first amplified from pMF164 (P$_{PIR}$-GFP expression construct) using oligonucleotides OMF87:

GATCACTAGTGATATCgctagctgtgtgtgagttc (SEQ ID NO:8) and OMF86:

35   GATCGGATCCATCGATAATAAAATATCTTTATTTTCATTACATCTGTGTGTTGGTTTTTTG

TGTGcgtcggagaaatagcgc (SEQ ID NO:9). OMF86 contains in its 5' extension an
artificial polyadenylation site (Levitt *et al.*, 1989, Genes Dev. 3: 1019-1025) optimized to
eliminate restriction sites. Also, OMF87 and OMF86 contain in their 5' extensions *Spe*I and
*Cla*I sites (bold), respectively. The P$_{PIR}$-pA-containing fragment was cloned into

5   pcDNA3.1/V5/His-TOPO (Invitrogen) in anti-P$_{CMV}$ orientation to give plasmid
pSAM224. The P$_{PIR}$-pA-containing cassette was released from pSAM224 by restriction
with *Spe*I and *Cla*I and cloned into the corresponding sites (*Spe*I/*Cla*I) of pTRIDENT1 to
replace *IRES*II to result in pDuoRex1. As shown in Fig. 6, pDuoRex1 consists of the
following double regulated expression unit: P$_{hCMV^*-1}$-MCSI-*IRES*-MCSII-pA$_I$-/-P$_{PIR}$-

10 MCSIII-pA$_{II}$ (P$_{hCMV^*-1}$, tetracycline-regulated promoter; MCS, multiple cloning site,
IRES, internal ribosomal entry site; *pA*, polyadenylation site; P$_{PIR}$, pristinamycin-
responsive promoter).

        Double regulation of Mek1DD, a constitutively active protein kinase of the central
mammalian growth factor signaling pathway and the cyclin-dependent kinase inhibitor

15 (CDI) p27 using pDuoRex1 was achieved by a 4-step cloning procedure: (1) Mek1DD was
amplified from (pGem-Mek1DD; Greulich and Erikson, 1998, J. Biol. Chem. 273:13280-
13288) by OMF92: GATCGATATC**ACTAGT**CGCCACCatgcccaagaagaagcc (SEQ ID
NO:10) and OMF93: GATC**GGATCC**ACGCGTtcagatgctggcagcgtg (SEQ ID NO:11)

20 and the 1.3 kb fragment was cloned into pcDNA3.1/V5/His-TOPO under control of the
P$_{CMV}$ promoter to give pMF192. (2) p27 was amplified (from pDD6; Fussenegger *et al.*,
1998) with OMF94: GATC**GAATTC**AAGCTTgcggtcgtgcagacccgg (SEQ ID NO:12)
and OMF95: ATGCATCGAT**GCGGCCGC**ttacgtttgacgtcttctg (SEQ ID NO:13) and
the 600 bp was cloned into pcDNA3.1/V5/His-TOPO under control of P$_{CMV}$ to give

25 pMF193. (3) p27 was excised from pMF193 by *Eco*RI and *Not*I (contained in OMF94 and
OMF95, respectively (bold)) and cloned into the corresponding sites of pDuoRex1
(*Eco*RI/*Not*I) thereby replacing the *IRES*-containing fragment and placing p27 under control
of P$_{hCMV^*-1}$ to result in plasmid pMF194. (4) pMF192 was restricted with *Spe*I and
*Bam*HI (contained in OMF92 and OMF93, respectively (bold)) and Mek1DD was

30 transferred to pMF194 linearized by *Spe*I and *Bgl*II thus placing Mek1DD under control of
P$_{PIR}$ to give plasmid pMF195. As shown in Fig. 7, pMF195 contains the following double
regulated expression unit: P$_{hCMV^*-1}$-p27-pA$_I$-/-P$_{PIR}$-Mek1DD-pA$_{II}$.

        Additional double regulation vectors analogous to pMF195 (which contained p27-
Mek1DD) were also constructed in which the Mek1DD gene coding sequence was replaced

35 by other growth promoting genes. Specifically constructs containing sequences encoding

the adenoviral large T antigen, the adenoviral small T antigen, and the human papillomavirus E7 protein under control of the P$_{PIR}$ promoter were constructed using well known techniques.

In a parallel cloning procedure pMF188 was cloned containing the P$_{hCMV*-1}$-
5   SEAP-*IRES*-pA$_I$-/-P$_{PIR}$-GFP-pA$_{II}$. GFP was amplified from pMF164 by OMF84:GATC**GCGGCCGC**AATAAAATATCTTTATTTTCATTACATCTGTGTGTTGGTTTTT TGTGTGcgtcggagaaatagcgc (SEQ ID NO:14) and OMF85: GTAC**AGATCT**tta tttgtagagctcatc (SEQ ID NO:15) and cloned into pcDNA3.1/V5/His-TOPO under
10 control of P$_{CMV}$ to give plasmid pSAM223. Since OMF84 contains in its extension an artificial pA element pSAM223 contains a pA-GFP cassette which was excised by *Not*I and *Bgl*II (contained in OMF84 and OMF85, respectively (bold)) and cloned into the corresponding sites (*Not*I/*Bgl*II) of pMF124 (pTRIDENT3 containing SEAP in the first cistron) to give pMF188.

15 A further double regulated configuration was cloned for use in transgenic animals utilizing the two compatible GFP-derived fluorescent proteins, CFP (cyan fluorescent protein) and YFP (yellowish fluorescent protein) were combined in a pDuoRex1-derived expression vector by a 4-step cloning procedure: (1) CFP was amplified from pECFP-C1 (Clontech) with oligos
20 OMF96:GATC**GAATTC**CCTCAGCACCAGGTCATGCTTAAGTCGCGACATATGgatccgcta gcgctaccg (SEQ ID NO:16) and OMF89: GATCAAGCTTGCCCGGGCCACACAAA AAACCAACACACAGATGTAATGAAAATAAAGATATTTTATTTGATCAGGCGCGCC**GCGGCC GCATGC**ttacttgtacagctcgtc (SEQ ID NO:17) and cloned into pcDNA3.1/V5/His-TOPO under control of P$_{CMV}$ to give plasmid pSAM228. (2) YFP was amplified from
25 pEYFP-C1 (Clontech) with oligos OMF90:GTACGAATTCGATATCATGCATGGCGCCGTTTAAACGCGTATTTAAATgatccgc tagcgctaccg (SEQ ID NO:18) and OMF91: GATCAAGCTTGCGGCCGCGGATCCG CCCGGGCCACACAAAAAACCAACACACAGATGTAATGAAAATAAAGATATTTTATTATCGA TACTAGTGCGATCGCTTAATTAATTTAAATttacttgtacagctcgtcc (SEQ ID NO:19)
30 and cloned into pcDNA3.1/V5/His-TOPO under control of P$_{CMV}$ to give plasmid pSAM222. (3). CFP was excised from pSAM228 by *Eco*RI and *Not*I (contained in OMF96 and OMF89, respectively (bold)) and cloned into the corresponding sites (*Eco*RI/*Not*I) of pDuoRex1 thereby replacing the *IRES* element to give pMF200. (4) pMF200 was linearized with *Eco*RV and *Spe*I ligated to YFP excised from pSAM222 by *Eco*RV and *Spe*I
35 (contained in OMF90 and OMF91, respectively (bold)) to give pMF201. As shown in Fig.

4, pMF201 contains the following double regulated expression unit: P_{hCMV^*-1}-CFP-pA$_I$-/-P$_{PIR}$-YFP-pA$_{II}$.

pMF196 is a CFP/YFP-containing double regulation construct similar to pMF201 but both expression units converge. Cloning of pMF196 required a 4-step cloning

5   procedure: (1) The YFP-pA containing cassette was excised from pSAM222 by *Eco*RI and *Not*I and ligated to the corresponding sites of pMF164 (*Eco*RI/*Not*I) thereby replacing GFP of pMF164 to result in plasmid pSAM226 (2) The P$_{PIR}$-YFP-pA cassette was excised with *Ssp*I/*Not*I from pSAM226 and ligated into the corresponding sites pTRIDENT 1 (*Ssp*I/*Not*I) thereby replacing P$_{hCMV^*-1}$ and *IRES* I of pTRIDENT1 to give

10   plasmid pMF190. (3) The CFP-pA cassette was excised from pSAM228 (OMF89 contains in it extension an artificial pA site) by *Eco*RI and *Hind*III (contained in OMF96 and OMF89, respectively) and ligated into the corresponding sites (*Eco*RI/*Hind*III) of pTBC1 (Dirks *et al.*, 1993) to result in plasmid pSAM227. (4) The P_{hCMV^*-1}-CFP-pA-containing cassette was released from pSAM227 by digestion with *Xho*I and *Srf*I and was ligated to

15   pMF190 digested with *Sal*I and *Srf*I to result in plasmid pMF196. As shown in Figure 4 pMF196 contains the following double regulated expression unit: P$_{PIR}$-CFP-pA$_I$/pA$_{II}$-YFP-P$_{hCMV^*-1}$.

RESULTS

Construction of tetracycline- and streptrogramin-responsive double regulation vectors

20   In order to demonstrate the double regulation concept a set of three vectors were constructed (i) pMF188 combining tetracycline-responsive SEAP (human secreted alkaline phosphatase) and pristinamycin-responsive GFP expression in a single double regulated expression unit: P$_{hCMV^*-1}$-SEAP-pA$_I$-P$_{PIR}$-GFP-pA$_{II}$ (Fig.3). (ii) Similar to pMF188, pMF201 contains tet-regulated CFP (cyan fluorescent protein) and pristinamycin-regulated

25   YFP (yellowish fluorescent protein) in a single expression unit: P$_{hCMV^*-1}$-CFP-pA$_I$8 -P$_{PIR}$-YFP-pA$_{II}$ (Fig. 4). (iii) pMF196 is isogenic to pMF188 and pMF201 but contains the following double regulated expression unit: P$_{PIR}$-YFP-pA$_I$-pA$_{II}$ -CFP-P$_{hCMV^*-1}$ (Fig. 5).

When these double regulation vectors were transiently transfected into CHO-TWIN1$_{108}$ cells all expression units contained on pMF188/pMF201/pMF196 showed

30

independent on/off regulation regulation in response to the respective regulating antibiotics tetracycline and pristinamycin as monitored by SEAP activity tests and fluorescence microscopy. Based on experience with pMF188/pMF201/pMF196 we designed a multipurpose double regulation vector pDuoRex1 for use in tissue engineering and gene

35   therapy (Fig. 6).

For the novel gene therapy and tissue engineering concept positive and negative proliferation control genes were carefully chosen. For negative proliferation control the use of tumor suppressor genes such as p21, p27, p53 or combinations thereof with differentiation factors such as *c/ebpα* and/or survival factors such as *bcl-2* and *bcl-x$_L$* in a

5   multicistronic configuration is well-tested and reliable (Fussenegger *et al.*, 1998). The choice of the positive proliferation control gene is important since its effect on engineered cells has to be sufficiently strong to induce resumption of the cell cycle in terminally differentiated cells but weak enough to allow reversion of the proliferating state and reimplantation of treated cells into the *in vivo* context.

10   We chose Mek1, a central protein kinase in the conserved mammalian Ras-MAP signal transduction pathway responding to growth-promoting signals such as cytokines. At the center of such signal transduction Mek1 not only transfers but also amplifies growth-promoting signals. Upstream kinases (Erk) phosphorylate Mek1 at two positions (serine 218) and (serine 222). A recently described double mutant contains two mutations which

15   alter these ser218 and ser22 to aspartate and render Mek1DD constitutively active in the absence of upstream proliferation signals and in the absence of phosphorylation (Greulich and Erikson, 1998, J. Biol. Chem. 273: 13280-13288). Previous results by Greulich and Erikson, 1998) have shown that regulated overexpression of Mek1DD enables proliferation of NIH-3T3 cells in low serum. A pDuoRex1-based expression vector, pMF195, was

20   constructed which contains p27 under control of P$_{hCMV*-1}$ and Mek1DD under control of P$_{PIR}$ (Fig. 7). Immunofluorescence analysis of CHO-TWIN1sub 108 transiently transfected with pMF195 show clear independent regulation of p27 and Mek1DD in response of the respective regulating antibiotic tetracycline and pristinamycin.

Besides Mek1DD other genetic determinants exerting positive control of

25   mammalian cell cycle such as cyclins (*e.g.* cyclin E) or E2F could also be envisioned. For example, double regulation vectors analogous to pMF195 (which contained p27-Mek1DD) were constructed in which the Mek1DD gene coding sequence was replaced by sequences encoding the adenoviral large T antigen, the adenoviral small T antigen, and the human papillomavirus E7 protein (all under control of the P$_{PIR}$ promoter). One can also use a

30   sequence encoding the human papillomavirus E6 protein. In principle, this two gene double regulation concept can also be adapted to the multicistronic level to carefully fine tune expression of opposing proliferation control genes or to combine, for example, growth-inhibiting genes with genes such as *c/ebpα* which induce and maintain terminal

35   differentiation. Also, monocistronic double regulation concepts could be foreseen which use sense and anti-sense expression of a single gene to induce or inhibit proliferation (or

vice versa). Candidate genes include p27 anti-sense, expression of which has been shown to stimulate proliferation of quiescent fibroblasts and enable growth in serum-free medium (Rivard *et al.*,1996, J. Biol. Chem. 271: 18337-18341.), and nedd5 which is known as positive growth controlling gene (Kinoshita *et al.*, 1997, Genes Dev. 11: 1535-1547 but

5 anti-sense expression may inhibit growth and leads to fried egg-like cell shapes as seen for cells arrested by overexpression of p27 (our own data not shown).

Example 3:   Promoter modification to enhance maximal expression from the
              streptogramin-responsive system

10   In principle, every part of the PIT/P_{PIR} system can be improved individually or exchanged by a more powerful component: (1) The Pip domain of PIT can be subjected to random mutagenesis to alter DNA binding affinity or specificity, to improve affinity to pristinamycin or other streptogramins or antibiotics or to reverse streptogramin responsiveness of the PIT/P_{PIR} system (reverse PIT/P_{PIR} system), for example allow

15 induction of gene expression upon addition of pristinamycin instead of its withdrawal. (2) The VP16 domain could be exchanged by other transactivation domains such as the p65 domain of human NF-κ B (Schmitz and Baeuerle, 1991, EMBO J. 10: 3805-3817) to "humanize" the regulation system and reduce immune recognition of PIT or the KRAB silencing domain of the human *kox1* gene (Deuschle *et al.*, 1995, Mol. Cell Biol. 15: 1907-

20 1914) to construct a reverse PIT/P_{PIR} system (these systems are under construction). (3) exchange of the minimal *Drosophila* heatshock promoter by other minimal promoters such as the minimal CMV promoter or the minimal promoter of the adenoviral E1B gene (also under construction).

          The naturally occurring Pptr sequence contains only three distinct Pip binding sites

25 which could accommodate PIT. In order to enhance maximal expression from streptogramin-responsive system, we constructed PI-responsive promoters with two (P_{PIR2}) and three (P_{PIR3}) ptr binding motifs with a total of 6 and 9 Pip binding sites, respectively.

          Two synthetic DNA fragments were synthesized:

PPIR2 (SEQ ID NO:20):

30 gacgtcgatatcGAAATAGCGCTGTACAGCGTATGGGAATCTCTTGTACGGTGTACGAGTA
TCTTCCCGTACACCGTACGAAATAGCGCTGTACAGCGTATGGGAATCTCTTGTACGGTGTA
CGAGTATCTTCCCGTACACCGTACcctgcagg and

PPIR3 (SEQ ID NO:21):

gacgtcgatatcGAAATAGCGCTGTACAGCGTATGGGAATCTCTTGTACGGTGTACGAGTA

35 TCTTCCCGTACACCGTACGAAATAGCGCTGTACAGCGTATGGGAATCTCTTGTACGGTGTA

CGAGTATCTTCCCGTACACCGTACGAAATAGCGCTGTACAGCGTATGGGAATCTCTTGTAC

GGTGTACGAGTATCTTCCCGTACACCGTAC**cctgcagg**.

Both DNA fragments were digested with *Aat*II and *Sse*8387I (corresponding restriction sites

are shown in bold) and ligated to the corresponding sites of pMF172 (P$_{PIR}$-SEAP;

5 *Aat*II/*Sse*8387I) thereby replacing the single Pip binding sequence (Pptr of *S.*

pristinaespiralis; Salah-Bey *et al.*, 1995; Salah-Bey and Thompson, 1995) to result in

plasmids pMF198 (P$_{PIR2}$-SEAP) and pMF199 (P$_{PIR3}$-SEAP).

All three PIT-dependent promoters P$_{PIR}$, P$_{PIR2}$ and P$_{PIR3}$ were cloned in front of

a *seap* reporter gene to measure their transcriptional activity in the absence of pristinamycin

10 (full induction). Table 8 shows relative expression profiles of P$_{PIR2}$ (pMF198; P$_{PIR2}$-

SEAP) and P$_{PIR3}$ (pMF199; P$_{PIR3}$-SEAP) compared to "wildtype" P$_{PIR}$.

Table 8: Relative SEAP Expression from Modified P$_{ptr}$-linked Genes

	SEAP expression in the absence of PI	SEAP expression in the presence of PI
P$_{PIR}$-SEAP (pMF172)	0.21 ± 0.01	0.04 ± 0.019
P$_{PIR2}$-SEAP (pMF198)	0.4 ± 0.05	0.073 ± 0.004
P$_{PIR2}$-SEAP (pMF199)	0.63 ± 0.009	0.09 ± 0.008

Example 4:   Construction of a pristinamycin-inducible expression system with enhanced
regulation characteristics

25   The Classical PIT/P$_{PIR}$ system belongs to the "OFF" family of regulation concepts

since gene expression is activated upon withdrawal of the regulating antibiotic. However,

in some applications such as gene therapy and tissue engineering an "ON" system which is

induced upon addition of streptogramin antibiotics is more desirable. We therefore

constructed a new Pip-based binary PI$_{ON}$ system which consists of a P$_{PIR}$ON promoter and a

30 set of two different transrepressors. P$_{PIR}$ON consists of a PIR3 module containing 3

consecutive Pip binding elements (SEQ ID NO:21; Example 3) placed in front of the strong

viral SV40 promoter. Transrepressors such as PIT3, which consists of a protein fusion

between Pip and the KRAB silencing domain of the human kox-1 gene (Deuschle et al.,

1995, Mol. Cell Biol. 15: 1907-1914) and PIT4, which is simply Pip expressed in a

35 eukaryotic configuration, bind to PIR3 in front of P$_{SV40}$ and block transcription of this

- 38 -

promoter. Besides sterical transcription blocking the silencing domain of PIT3 can additionally downregulate P_{SV40} activity.

MATERIALS AND METHODS

The PIR3 sequence (SEQ ID NO:20) was excised from GeneOp™-PIR3 (the pUC-
5 derived GeneOp expression vector is sold by Operon Technologies Inc.) by *Eco*RV and *Sma*I and ligated into the *Nru*I site of pSEAP2-control (Clontech) thus resulting in plasmid pMF208 containing P_{SV40}-PIR3-SEAP cassette. The combination of P_{SV40} and PIR3 is designated P_{PIR}ON.

PIT3, the Pip-KRAB fusion protein was constructed by a three step cloning
10 procedure: (i) The KRAB domain of the human *kox*-1 gene was amplified from pSCTEVgal93Kox (Moosmann et al., 1997, Biol. Chem. 378: 669-677) with oligos OMF99:GATC**GCGCGC**C (AGATCCAAAAAAGAAGAGAAAGGT) (AG ATCCAAAAAGAAGAGAAAGGT) (AGATCCAAAAAAGAAGAGAAAGGT) AATGGATGCTAAG TCACTA (SEQ ID NO:25) and OMF100: GATC<u>AAGCTT</u>GGATCCTTACC
15 AGAGATCATTCCTTGC (SEQ ID NO:26) and ligated in antisense orientation into pcDNA3.1/V5/His-TOPO (Invitrogen) to result in pMF203. OMF99 contains three consecutive nuclear localization signals (bracketed) derived from the SV40 large-T-antigen (Kalderon et al., 1984). (ii) The KRAB domain was excised from pMF203 by *Bss*HII/*Hind*III and ligated to the corresponding sites (*Bss*HII/*Hind*III) of pSBC2-tTA
20 (Fussenegger *et al.*, 1997) thus replacing the VP16 domain of tTA and resulting in pMF205. (iii) The *tet*R domain was excised of pMF205 by *Ssp*I/*Bss*HII and replaced by Pip excised from pMF150 by *Ssp*I/*Bss*HII to give plasmid pMF207. The resulting plasmid has the following PIT3 coding sequence:

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ATGAGTCGAGGAGAGGTGCGCATGGCGAAGGCAGGGCGGGAGGGGCCGCGGGACAGCGTGT
GGCTGTCGGGGGAGGGGCGGCGCGGCGGTCGCCGTGGGGGGCAGCCGTCCGGGCTCGACCG
GGACCGGATCACCGGGGTCACCGTCCGGCTGCTGGACACGGAGGGCCTGACGGGGTTCTCG
ATGCGCCGCCTGGCCGCCGAGCTGAACGTCACCGCGATGTCCGTGTACTGGTACGTCGACA
CCAAGGACCAGTTGCTCGAGCTCGCCCTGGACGCCGTCTTCGGCGAGCTGCGCCACCCGGA
CCCGGACGCCGGGCTCGACTGGCGCGAGGAACTGCGGGCCCTGGCCCGGGAGAACCGGGCG
CTGCTGGTGCGCCACCCCTGGTCGTCCCGGCTGGTCGGCACCTACCTCAACATCGGCCCGC
ACTCGCTGGCCTTCTCCCGCGCGGTGCAGAACGTCGTGCGCCGCAGCGGGCTGCCCGCGCA
CCGCCTGACCGGCGCCATCTCGGCCGTCTTCCAGTTCGTCTACGGCTACGGCACCATCGAG
GGCCGCTTCCTCGCCCGGGTGGCGGACACCGGGCTGAGTCCGGAGGAGTACTTCCAGGACT
CGATGACCGCGGTGACCGAGGTGCCGGACACCGCGGGCGTCATCGAGGACGCGCAGGACAT
CATGGCGGCCCGGGGCGGCGACACCGTGGCGGAGATGCTGGACCGGGACTTCGAGTTCGCC
CTCGACCTGCTCGTCGCGGGCATCGACGCGATGGTCGAACAGGCCTCCGCGTACAGCCGCG
CGCCAGATCCAAAAAAGAAGAGAAAGGTAGATCCAAAAAAGAAGAGAAAGGTAGATCCAAA
AAAGAAGAGAAAGGTAATGGATGCTAAGTCACTAACTGCCTGGTCCCGGACACTGGTGACC
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TTCAAGGATGTATTTGTGGACTTCACCAGGGAGGAGTGGAAGCTGCTGGACACTGCTCAGC
AGATCGTGTACAGAAATGTGATGCTGGAGAACTATAAGAACCTGGTTTCCTTGGGTTATCA
GCTTACTAAGCCAGATGTGATCCTCCGGTTGGAGAAGGGAGAAGAGCCCTGGCTGGTGGAG
AGAGAAATTCACCAAGAGACCCATCCTGATTCAGAGACTGCATTTGAAATCAAATCATCAG
TTTCCAGCAGGAGCATTTTTAAAGATAAGCAATCCTGTGACATTAAAATGGAAGGAATGGC
5 AAGGAATGATCTCTGGTAA (SEQ ID NO:27).

For construction of PIT4, the Pip protein of *Streptomyces coelicolor* was amplified
from pGemT::Epip4 with oligos OMF63:GTACGAATTCCCACCATGAGTCGAGGAGAGGTG
(SEQ ID NO:1) and OMF103: GATCAAGCTTTCAGGCCTGTTCGACCATC (SEQ ID NO:28)
followed by cloning into vector pcDNA3.1/V5/His-TOPO (Invitrogen) under control of

10 P_{CMV}. The resulting plasmid pMF225 contains the following PIT4 sequence which
corresponds to the sequence of the pip gene of *Streptomyces coelicolor*:
ATGAGTCGAGGAGAGGTGCGCATGGCGAAGGCAGGGCGGGAGGGGCCGCGGGACAGCGTGT
GGCTGTCGGGGGAGGGGCGGCGCGGCGGTCGCCGTGGGGGGCAGCCGTCCGGGCTCGACCG
GGACCGGATCACCGGGGTCACCGTCCGGCTGCTGGACACGGAGGGCCTGACGGGGTTCTCG
ATGCGCCGCCTGGCCGCCGAGCTGAACGTCACCGCGATGTCCGTGTACTGGTACGTCGACA
15 CCAAGGACCAGTTGCTCGAGCTCGCCCTGGACGCCGTCTTCGGCGAGCTGCGCCACCCGGA
CCCGGACGCCGGGCTCGACTGGCGCGAGGAACTGCGGGCCCTGGCCCGGGAGAACCGGGCG
CTGCTGGTGCGCCACCCCTGGTCGTCCCGGCTGGTCGGCACCTACCTCAACATCGGCCCGC.
ACTCGCTGGCCTTCTCCCGCGCGGTGCAGAACGTCGTGCGCCGCAGCGGGCTGCCCGCGCA
CCGCCTGACCGGCGCCATCTCGGCCGTCTTCCAGTTCGTCTACGGCTACGGCACCATCGAG
20 GGCCGCTTCCTCGCCCGGGTGGCGGACACCGGGCTGAGTCCGGAGGAGTACTTCCAGGACT
CGATGACCGCGGTGACCGAGGTGCCGGACACCGCGGGCGTCATCGAGGACGCGCAGGACAT
CATGGCGGCCCGGGGCGGCGACACCGTGGCGGAGATGCTGGACCGGGACTTCGAGTTCGCC
CTCGACCTGCTCGTCGCGGGCATCGACGCGATGGTCGAACAGGCCTGA (SEQ ID NO: 29).

pMF150 is almost identical to pMF225 but the TGA stop codon of Pip encoded on
pMF150 has been mutated in order to allow fusion to the various transactivating and
25 transrepressing domains. Because of the mutation of this stop codon the *pip* gene contained
in pMF150 is terminated a few bp downstream at the next stop codon encountered in the
vector sequence. However, both Pip proteins perform equally in the PipON regulation
concept.

RESULTS
30 *Regulation characteristics of the PipON system*

CHO-K1 cells were cotransfected with pMF208 (P_{PIR}ON-SEAP) and either
transrepressor-containing plasmid pMF207 (PIT3; Pip-KRAB) or pMF225 (PIT4; Pip).
Cells were grown in the absence and presence of PI. In the absence of PI, expression of
P_{PIR}ON was fully repressed and induction could be observed in the presence of PI. Table 9
35 shows the regulation characteristics of both inducible regulation concepts.

	No Inducer	+ PI (2µg/ml)
CHO + pMF208 + pMF207	0.08 ± 0.006	0.00055 ± 0.0003
CHO + pMF208 + pMF225	0.65 ± 0.03	0.019 ± 0.002
CHO + pSEAP2-control	0.67 ± 0.04	

Table 9: Comparison of the regulation characteristics of the PipON systems. CHO-K1 cells were simultaneously cotransfected with the plasmids indicated and the SEAP readout (slope of the p-nitrophenolphosphate-based light absorbance time courses) was determined in the presence and absence of pristinamycin 48 hr post transfection. The SEAP expression levels of the PipON systems were compared to the pSEAP2-control construct which is isogenic to pMF208 but lacks the PIR3 binding element in front of the P_{SV40} promoter.

While both PipON systems are induced in the presence of PI, their maximal as well as their basal expression levels differ significantly. Whereas the PIT4-P_{PIR}ON configuration (pMF225-pMF208) reaches expression levels comparable to the strong constitutive viral SV40 promoter and shows an induction factor of 25 which is more than double the induction factor of the classical PIT/P_{PIR} system, the PIT3-P_{PIR}ON configuration resulted in the tightest repression and an induction factor of about 150, more than 15 times higher compared to the PIT/P_{PIR} system. However, the maximal expression level of the PIT-P_{PIR}ON configuration is about 3-fold lower compared to the maximal expression level of the PIT/P_{PIR} configuration. The difference in their regulation characteristics enables the use of the two PIPON system for a broad range of different applications. In configurations which require high expression levels the use of the PIT4/P_{PIR}ON configuration is advantageous whereas situations which require tightest repression of basal expression of the transgene, the PIT3/P_{PIR}ON concept is the preferred system.

The modular setup of P_{PIR}ON consisting of an independent operator sequence (PIR3) and a fully functional promoter element allows straightforward exchange of PSV40 of P_{PIR}ON by any type of promoter to enable, for example, tissue-specific regulated expression or adaptation of this inducible regulation concept to other organisms such as yeast and plants.

Induction potential of different streptogramin antibiotics on the Pip/P$_{PIR}$ON regulation system

In order to assess the regulation potential of different streptogramin antibiotics, pMF225 or pMF150 encoding PIT4 were cotransfected with pMF208 (P$_{PIR}$ON-SEAP) into
5   CHO-K1 cells and the SEAP induction was measured 48 following transfection (as described above in Example 1) and addition of the following streptogramin antibiotics: (i) PI, the group B streptogramin component of pristinamycin; (ii) Pyostacin, the human oral antibiotic; (iii) Synercid, the first injectable semi-synthetic pristinamycin derivative effective against most multidrug resistant human pathogenic bacteria and (iv) virginiamycin
10 used as growth promotant in livestock feed. 2µg/ml group B component was used for the regulation studies (for mixed streptogramins the concentration of the group B component was determined based on the fixed 70%:30% ratio of group A and group B components).

Table 10 shows the induction potential of the different streptogramins. Pyostacin is as efficient in inducing the Pip/P$_{PIR}$ON as is pure PI component, virginiamycin exhibits
15 slightly lower regulation performance and Synercid has almost no inducing capacity on the Pip/P$_{PIR}$ON system. Therefore, the use of Synercid in antibiotic chemotherapy is likely to be compatible with the use of the Pip/P$_{PIR}$ON system in gene therapy and tissue engineering applications.

	SEAP Production
No Streptogramin	0.019 ± 0.002
Pristinamycin I	0.65 ± 0.03
Pyostacin	0.70 ± 0.005
Synercid	0.036 ± 0.0014
Virginiamycin	0.4125 ± 0.05

Table 10: Induction performance of different streptogramin antibiotics. CHO-K1 cells were simultaneously cotransfected with the plasmids pMF225 (PIT4) and pMF208 (P$_{PIR}$ON-SEAP) and the SEAP readout (slope of the p-nitrophenolphosphate-based light absorbance time courses) was determined in the presence and absence of the streptogramins indicated
5 48 hr post transfection.

Example 5:   Construction of multi-purpose expression vectors

In order to use the pristinamycin-responsive mammalian gene regulation system in a wide variety of applications, a set of 6 mammalian gene expression vectors which are
10 compatible with the use of all pristinamycin-dependent transactivators (PIT, PIT2) and transrepressors (PIT3, PIT4) was constructed. The first set of vectors, pMF189 and pMF229, consists of monocistronic expression vectors containing the P$_{PIR}$ (pMF189) and P$_{PIR}$ON (pMF229) promoters followed by a multiple cloning site of up to 22 unique restriction sites 6 of which are rare-cutting sites for enzymes recognizing 8 bp (Figure 8).
15   The second set of vectors is shown in Figure 9 and contains the same promoters P$_{PIR}$ and P$_{PIR}$ON integrated in the pTRIDENT family of tricistronic expression vectors (Fussenegger *et al.* 1998, Biotechnol. Bioeng. 57,1-10). pTRIDENT vectors contain a single tricistronic expression unit which is driven either by P$_{PIR}$ (pTRIDENT9 and pTRIDENT10) or P$_{PIR}$ON (pTRIDENT11 and pTRIDENT12). While the first cistron is
20 translated in the classical cap-dependent manner, the following two cistrons rely on cap-independent translation initiation mediated by internal ribosomal binding sites of the encephalomyocarditis virus or of polioviral origin (*IRES*). While pTRIDENT9 (P$_{PIR}$) and pTRIDENT11 (P$_{PIR}$ON) contain two *IRES* elements, pTRIDENT10 (P$_{PIR}$) and pTRIDENT 12 (P$_{PIR}$ON) contain an *IRES* as well as a *CITE* element. Both *IRES* elements are among the
25 strongest currently available, showing high translation initiation in a wide variety of mammalian cells and tissues (Borman et al., 1997; Fussenegger *et al.*, 1998, Biotechnol. Bioeng. 57,1-10, Fussenegger *et al.*, 1998, Nat. Biotechnol. 16,468-472). Both *IRES* elements are flanked by large polylinkers which allow convenient movement of genes into pTRIDENT derivatives. pTRIDENT vectors have proven to be useful tools for a wide
30 variety of applications (Fussenegger *et al.*, 1998, Biotechnol. Bioeng. 57,1-10; Fussenegger *et al.*, 1998, Nat. Biotechnol. 16, 468-472)

Construction of multi-purpose expression vectors

pMF189 the P$_{PIR}$ expression vector was constructed by a three step cloning procedure: (i) the yellowish fluorescent protein YFP was amplified from pEYFP-C1
35 (Clontech) with oligos OMF90:

GTACGAATTCGATATCATGCATGGCGCCGTTTAAACGCGTATTTAAATGATCCGCTAGCGC
TACCG (SEQ ID NO:30) and

OMF91:GATCAAGCTTGCGGCCGCGGATCCGCCCGGGCCACACAAAAAACCAACACACAGA
TGTAATGAAAATAAAGATATTTTATTATCGATACTAGTGCGATCGCTTAATTAATTTAAAT
TTACTTGTACAGCTCGTCC (SEQ ID NO:31) and ligated into pcDNA3.1/V5/His-TOPO to
result in pSAM222. (ii) YFP was excised from pSAM222 by $EcoRI/NotI$ and ligated to
pMF164 (cut with $EcoRI/NotI$) thereby replacing GFP of pMF164 (P$_{PIR}$-GFP) and resulting
in pSAM226 (P$_{PIR}$-YFP). (iii) YFP was eliminated from pSAM226 by $SwaI$ and the
remaining vector was religated to result in plasmid pMF189.

pMF229 was constructed by a three-step cloning procedure: (i) The PIR3 element
was excised from pMF208 by $XhoI/EcoRI$ and ligated to pTBC1 ($XhoI/EcoRI$) thereby
replacing the tetracycline-responsive promoter P$_{hCMV*-1}$ of pTBC1 and resulting in pMF210.
(ii) pMF210 was restricted with $SspI/StuI$ and the viral SV40 promoter excised from pSBC1
with $SspI/StuI$ was cloned in front of the PIR3 element to reconstitute P$_{PIR}$ON and result in
plasmid pMF222. (iii) The P$_{PIR}$ON promoter was subsequently excised from pMF222 by
$SspI/EcoRI$ and ligated to pMF189 restricted with $SspI/EcoRI$ thereby replacing P$_{PIR}$ of
pMF189 with P$_{PIR}$ON and resulting in pMF229.

For construction of the tricistronic pTRIDENT expression vectors the P$_{PIR}$ promoter
was excised from pMF164 by $SspI/EcoRI$ and the P$_{PIR}$ON promoter was excised from
pMF222 by $SspI/EcoRI$.

The P$_{PIR}$ promoter elements were subsequently cloned:

1. into the $SspI/EcoRI$ sites of pTRIDENT1 to replace P$_{hCVM*-1}$ and result in pTRIDENT 9
(P$_{PIR}$-MCSI-IRESI-MCSII-IRESII-MCSIII-pA).

2. into the $SspI/EcoRI$ sites of pTRIDENT3 P$_{hCVM*-1}$ and result in pTRIDENT 10 (P$_{PIR}$-
MCSI-IRES-MCSII-CITE-MCSIII-pA).

The P$_{PIR}$ON promoter elements were subsequently cloned:

1. into the $SspI/EcoRI$ sites of pTRIDENT1 to replace P$_{hCVM*-1}$ and result in pTRIDENT 11
(P$_{PIR}$ON-MCSI-IRESI-MCSII-IRESII-MCSIII-pA).

2. into the $SspI/EcoRI$ sites of pTRIDENT3 to replace P$_{hCVM*-1}$ and result in pTRIDENT 12
(P$_{PIR}$ON-MCSI-IRES-MCSII-CITE-MCSIII-pA).

*Construction of the positive feedback regulation system using the streptogramin-responsive
regulation concept*

In contrast to the classical PIT/P$_{PIR}$ system in which PIT and P$_{PIR}$ reside on different
plasmids, the positive feedback regulation concept places both elements in a single, often
mutlticistronic, expression unit. In particular, the transactivator PIT is placed under control
of its target promoter P$_{PIR}$.

In this configuration initial transcripts originating from the leakiness of the P_{PIR} promoter lead to few PIT molecules which are inactivated in the presence of stregtogramins. However, in the absence of this class of antibiotics initial PIT molecules can bind to and induce P_{PIR}. Since a PIT transcript is produced in every round of transcription, a principle

5   called positive feedback, PIT accumulates in the cell and ensures high-level expression of the transgene of interest, yet this system retains full regulatability. Advantages of the positive feedback regulation system over classical binary regulated expression systems are:

1.   Tighter repression of gene expression since PIT is not expressed constitutively but originates from rare leaky transcripts. Therefore, in the repressed situation (in the

10   presence of pristinamycin) little PIT is present in the cell which initiate transcription from P_{PIR} in contrast to the situation in which PIT is constitutively expressed from a separate vector.

2.   The positive feedback system produces a PIT molecule in every round of transcription leading to higher intracellular PIT levels and therefore also higher

15   expression of the transgene of interest.

3.   The positive feedback regulation concept establishes regulated gene expression in a single step. The classical binary PIT/P_{PIR} expression systems requires first installation of PIT and then installation of the P_{PIR}-responsive gene. Two subsequent rounds of transfection and selection is not only tedious and time consuming but also

20   undesired for advanced future therapies such as tissue engineering and gene therapy since the genome is changed significantly more than in a one-step engineering approach.

These positive feedback regulation vectors that were constructed contain both the green fluorescent protein GFP and PIT in a dicistronic, P_{PIR}-driven configuration. When

25   pMF170 (PPIR-GFP-IRES-PIT-pA) was transfected in CHO-K1, BHK-21 or HeLa cells bright green fluorescence could be observed by fluorescence microscopy in the absence of PI whereas GFP-expression was completely repressed in the presence of PI.

Also constructed was pTRIDENT-PIT (pMF175), which contains the pristinamycin-dependent transactivator in the first cistron of pTRIDENT9. Cistrons 2 and 3 of

30   pTRIDENT-PIT could accommodate two different genes of interest. Therefore, pTRIDENT-PIT derivatives enable one-step installation of streptogramin-responsive expression of up to two independent genes.

Construction of the positive feedback regulation vectors

For construction of the autoregulatory system, PIT was excised from pMF156 by

35   *EcoRI/HindIII* and cloned into the polylinker of the eukaryotic expression vector pSBC-2

(*Eco*RI/*Hind*III). As a pSBC-2 derivative, the resulting plasmid pMF169 is compatible to
the pSBC-1 derivative pMF164 (P$_{PIR}$-GFP) and allows fusion of both expression units via
their *Ssp*I/*Not*I restriction sites, thereby generating pMF170. pMF170 contains a dicistronic
P$_{PIR}$-regulated expression unit encoding GFP in the first and PIT in the second cistron. Cap-

5   independent translation initiation of the second cistron is enabled by a picornaviral internal
ribosomal entry site (*IRES*) encoded upstream of PIT.

For construction of pTRIDENT-PIT, PIT was excised from pMF156 by
*Eco*RI/*Hind*III and cloned into pTRIDENT 1 was restricted with *Eco*RI/*Hind*III and PIT to
result in plasmid pMF168. pMF168 was then restricted with *Ssp*I/*Eco*RI and the P$_{hCMV^\ast-1}$

10 was replaced by P$_{PIR}$ excised from pMF164 (P$_{PIR}$-GFP) with *Ssp*I/*Eco*RI to result in pMF175
which is designated pTRIDENT-PIT.

Example 6:   Detection of novel antibiotic activities using the streptogramin-responsive
                      expression technology

15   Streptogramins are unique antibiotics. Due to their composite nature (group A and
group B streptogramins), they are less likely to elicit resistance mechanisms and show
higher bacteriocidal acitivity because of a synergistic effect of both compounds. For
example, one particularly useful streptogramin antibiotic is Synercid®, a semi-synthetic,
injectable pristinamycin derivative, which is active against a wide variety of multidrug

20 resistant human pathogenic bacteria including vancomycin-resistant species. Infections of
these vancomycin-resistant specie are causing increasing health concerns in both the US and
Europe.

A sensitive streptogramin detection technology will have two major impacts on
current environmental and medical concerns: (i) Detection of trace amounts of banned

25 streptogramins such as virginiamycin in the human food chain for inspection purposes and
(ii) detection of novel streptogramin antibiotics in the culture supernatants of Actinomycetes
and fungi.

RESULTS

The streptogramin detection technology is based on the pristinamycin-inducible

30 (PipON) system (although the pristinamycin-repressible version can also be used) which has
been established in mammalian cells (CHO-K1) stably or by cotransfection of pMF150 or
pMF225 (Pip expression vector) and pMF208 (P$_{PIR}$ON-SEAP). The assay is based on the
fact that presence of streptogramins in the test sample will induce the P$_{PIR}$ON promoter and
lead to a quantitative change in the SEAP readout.

35   Using 100 microliters of *Streptomyces pristinaespiralis* (a major producer of

pristinamycin) supernatant as standard, we compared the detection potential of the
pristinamycin-responsive system to classical antibiogram tests using a collection of
streptogramin-sensitive human pathogenic bacteria including *Staphylococcus aureus*,
Streptococcus pneumoniae, *Streptococcus pyogenes*, *Listeria monocytogenes*,

5   *Corynebacterium diphtheriae*, *Haemophilus influenzae* and *Neisseria gonorrhoeae* as well
as the non-pathogenic *Bacillus subtilis* which is routinely used to screen for novel
antibiotics effective against Gram positive bacteria. The presence of pristinamycin could be
reliably detected in *S. pristinaespiralis* supernatants using the PipON system which resulted
in about 20% of the SEAP readout typically reached using 2 μg/ml of pure pristinamycin I.

10   However, using the same amount of supernatant in antibiogram tests, antibiotic activity
could only be detected in the hypersensitive strain of *C. diphtheriae* (19 mm of inhibitory
diameter). All other pathogens tested including the standard antibiogram reference strain *B.
subtilis* failed to detect pristinamycin in the *S. pristinaespiralis* supernatant despite of the
synergy of streptogramins which has been reported to increase the bacteriocidal activity by

15   a factor of 100 (Cocito *et al.*, 1997, J. Antimicrob. Chemother. 39: 7-13).

Following efforts to detect new streptogramin derivatives we tested the culture
supernatants of 14 novel *Streptomyces* isolates for the presence of antibiotic activity.
Antibiogram analysis of the supernatants using *S. aureus*, *S. pyogenes*, *L. monocytogenes*,
C. diphteriae, and *N. gonorrhoeae*, and 10 ml of culture supernatant, only two supernatants

20   showed an antibiotic profile typical for streptogramins. One of these supernatants also
induced the SEAP readout of the PipON system about 3-fold using only 100 microliters of
supernatant which shows that the Pip-based streptogramin detection technology outperforms
classical antibiogram test by at least a factor of 100. The second supernatant which was
positive in the antibiogram test decreased the cell viability when applied to the PipON

25   setup, indicating the potential antibiotic is cytotoxic to human cells. The correlation
between antibiotic acitivity and induction of SEAP suggests that a novel *Streptomyces*
species has been isolated which produces a streptogramine-like antibiotic activity.

The screening technology using the pristinamycin-responsive expression strategy
has the following advantages over classical antibiogram tests: (i) Antibiotic screening is not

30   limited by the sensitivity of indicator bacteria to a yet uncharacterized streptogramin
(sensitivity of bacteria to antibotics greatly varies between strains and even isolates), (ii) the
mammalian cell-based streptogramin detection concept shows at least two order of
magnitude higher sensitivity to this class of antibiotics than antibiogram tests based on

35   bacteriocidal activity, (iii) the mammalian screening context ensures exclusive detection of
bioavailable and non-cytotoxic antibiotics.

The foregoing written specification is sufficient to enable one skilled in the art to practice the invention. Indeed, various modifications of the above-described means for carrying out the invention which are obvious to those skilled in the field of molecular biology, medicine or related fields are intended to be within the scope of the following

5   claims. All references cited herein are incorporated by reference.

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What is claimed is:

1.   An isolated nucleic acid encoding a polypeptide which binds to a P$_{abr}$ sequence in the absence of its cognate antibiotic, wherein the nucleic acid hybridizes under moderate

5   stringency conditions to a sequence that encodes the polypeptide encoded by SEQ ID NO:29.

2.   The isolated nucleic acid of claim 1, wherein the nucleic acid hybridizes under high stringency conditions to a sequence that encodes the polypeptide encoded by SEQ ID

10   NO:29.

3.   The isolated nucleic acid of claim 1, wherein the nucleic acid hybridizes under moderate stringency conditions to the sequence of SEQ ID NO:29.

15   4.   The isolated nucleic acid of claim 1, wherein the nucleic acid hybridizes under high stringency conditions to the sequence of SEQ ID NO:29.

5.   The isolated nucleic acid of claim 1, wherein the polypeptide which binds to the P$_{abr}$ sequence is derived from a bacterium selected from the group consisting of *Actinomycetes*.

20

6.   The isolated nucleic acid of claim 5, wherein the *Actinomycete* is a *Streptomycete*.

7.   A substantially purified or isolated protein encoded by the nucleic acid of claim 1.

25
8.   A host cell containing the nucleic acid of claim 1.

9.   The host cell of claim 8, further comprising a nucleotide sequence to be transcribed operatively linked to eukaryotic promoter and a P$_{abr}$ sequence.

30
10.   An isolated nucleic acid encoding a polypeptide which binds to a P$_{abr}$ sequence in the absence of its cognate antibiotic operatively linked to a second polypeptide which activates or represses transcription in eukaryotic cells.

35   11.   The isolated nucleic acid of claim 10, wherein the polypeptide that activates

- 49 -

transcription is selected from the group consisting of a VP16 activating domain, a GAL4 activating domain, a CTF/NF1 activating domain, an AP2 activating domain, an ITF1 activating domain, an ITF2 activating domain, an Oct1 activating domain, a Sp1 activating domain, and the p65 domain of NF-κB.

5

12.   The isolated nucleic acid of claim 10, wherein the polypeptide that represses transcription is selected from the group consisting of a v-erbA oncogene product repressor domain, a thyroid hormone receptor repressor domain, a retinoic acid receptor repressor domain, a Drosophila Krueppel (Kr) protein repressor domain, a KRAB domain of the kox1

10 gene family, an even-skipped repressor domain, an *S. cerevisiae* Ssn6/Tup1 protein complex, a yeast SIRI protein, NeP1, a Drosophila dorsal protein, TSF3, SF1, a Drosophila hunchback protein, a Drosophila knirps protein, a WT1 protein, an Oct-2.1 repressor domain, a Drosophila engrailed protein, E4BP4, ZF5, a 65 amino acid repressor domain of E4BP4 and an N-terminal zinc finger domain of ZF5.

15

13.   The isolated nucleic acid of claim 10, wherein the polypeptide which binds to the P_{abr} sequence is derived from a bacterium selected from the group consisting of *Actinomycetes*.

20

14.   The isolated nucleic acid of claim 13, wherein the *Actinomycete* is a *Streptomycete*.

15.   A substantially purified or isolated protein encoded by the nucleic acid of claim 10.

25 16.   A host cell containing the nucleic acid of claim 10.

17.   The host cell of claim 16, further comprising a nucleotide sequence to be transcribed operatively linked to a P_{abr} sequence.

30 18.   The host cell of claim 17, wherein the nucleotide sequence to be transcribed is endogenous to the host cell.

19.   The host cell of claim 17, wherein the nucleotide sequence to be transcribed is exogenous to the host cell.

35

20.   The host cell of claim 17, wherein the nucleotide sequence to be transcribed is a

reporter gene.

21.   The host cell of claim 20, wherein the reporter gene is SEAP or GFP.

5   22.   An isolated nucleic acid comprising a P_{abr} sequence operatively linked to a first
eukaryotic promoter.

23.   The isolated nucleic acid of claim 22, wherein the first eukaryotic promoter is
operatively linked to a first coding sequence.
10
24.   The isolated nucleic acid of claim 22, further comprising a second eukaryotic
promoter.

25.   The isolated nucleic acid of claim 24, wherein at least one tet operator sequence is
15 operatively linked to the second eukaryotic promoter.

26.   The isolated nucleic acid of claim 24, wherein the second eukaryotic promoter is
operatively linked to a second coding sequence.

20
27.   The isolated nucleic acid of claim 23 or 26, wherein at least the first or the second
coding sequence contains an internal ribosomal entry site (IRES).

28.   The isolated nucleic acid of claim 27, wherein at least the first or the second coding
sequence encodes a P_{abr} -binding protein.
25

29.   The isolated nucleic acid of claim 26, wherein either the first coding sequence or the
second coding sequence encodes at least a tumor suppressor gene product.

30.   The isolated nucleic acid of any one of claims 24-26 and 29, wherein either the first
30 coding sequence or the second coding sequence encodes at least one gene product which
activates cell proliferation.

31.   The isolated nucleic acid of claim 29, further comprising a sequence encoding a
survival factor.
35

32.   A host cell genetically engineered to contain a nucleic acid comprising a P_{abr} sequence operatively linked to a first eukaryotic promoter.

33.   A method for regulating expression of a P_{abr} -linked gene in a eukaryotic cell,
5   comprising introducing into the cell a nucleic acid molecule encoding a P_{abr} -binding protein, thereby rendering the P_{abr} -linked gene capable of regulation by an antibiotic that binds to the P_{abr} -binding protein.

34.   The method of claim 33, wherein the P_{abr} -binding protein further comprises an
10 operably linked second polypeptide that activates or represses transcription in eukaryotic cells.

35.   The method of claim 33, further comprising modulating the level of the antibiotic in the cell.
15

36.   The method of claim 33, wherein the P_{abr} -binding protein is derived from
Amycolatopsis mediterranei, Streptomyces peucetius, Streptomyces cyanogenus, Streptomyces hygroscopicus, Streptomyces glaucescens, or *Streptomyces coelicolor.*

20
37.   The method of claim 34, wherein the polypeptide that activates transcription is selected from the group consisting of a VP16 activating domain, a GAL4 activating domain, a CTF/NF1 activating domain, an AP2 activating domain, an ITF1 activating domain, an ITF2 activating domain, an Oct1 activating domain, a Sp1 activating domain, and the p65 domain of NF-κB.
25

38.   The method of claim 34, wherein the polypeptide that represses transcription is selected from the group consisting of a v-erbA oncogene product repressor domain, a thyroid hormone receptor repressor domain, a retinoic acid receptor repressor domain, a
30 Drosophila Krueppel (Kr) protein repressor domain, a KRAB domain of the kox1 gene family, an even-skipped repressor domain, an *S. cerevisiae* Ssn6/Tup1 protein complex, a yeast SIRI protein, NeP1, a Drosophila dorsal protein, TSF3, SF1, a Drosophila hunchback protein, a Drosophila knirps protein, a WT1 protein, an Oct-2.1 repressor domain, a Drosophila engrailed protein, E4BP4, ZF5, a 65 amino acid repressor domain of E4BP4 and
35 an N-terminal zinc finger domain of ZF5.

39.   A process for producing a protein comprising:

culturing a eukaryotic cell containing:

a P_{abr}-linked gene that encodes the protein; and

a nucleic acid molecule encoding a P_{abr}-binding protein, and regulating expression

5   of the P_{abr}-linked gene by modulating the level of an antibiotic that binds to the P_{abr}-

binding protein in the cell.

40.   The process of claim 39, further comprising the step of collecting the protein

produced by the cell.

10

41.   The process of claim 39 or 40, wherein the P_{abr}-binding protein further comprises an

operably linked polypeptide that activates or represses transcription in eucaryotic cells.

42.   A method for screening for a candidate antibiotic, the method comprising

15   incubating a host cell in the presence of a test compound,

wherein the host cell comprises a polypeptide which binds to a P_{abr} sequence in the absence

of its cognate antibiotic and a nucleotide sequence encoding a reporter gene operatively

linked to a P_{abr} sequence, and wherein a change in the transcription of the reporter gene

indicates that the test compound is a candidate antibiotic.

20

43.   The method of claim 42, wherein the host cell is a mammalian host cell.

44.   The method of claim 42, wherein the host cell is a microbial host cell.

25

45.   The method of claim 44, wherein the host cell is a prokaryotic host cell.

46.   The method of claim 42, wherein the polypeptide which binds to the P_{abr} sequence is

derived from an *Actinomycetes* bacterium.

30

47.   The method of claim 46, wherein the *Actinomycete* is a *Streptomycete*.

48.   The method of claim 46, wherein the polypeptide which binds to the P_{abr} sequence is

the pristinamycin induced protein from *Streptomyces coelicolor*.
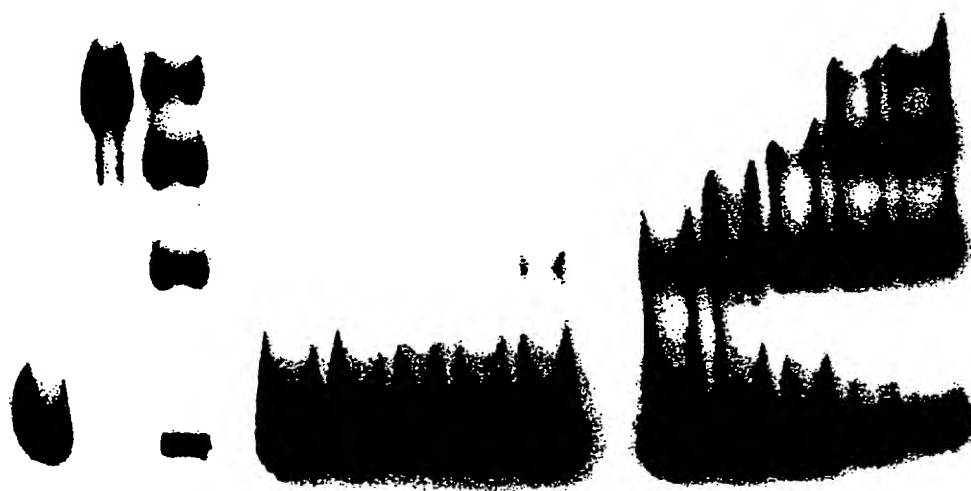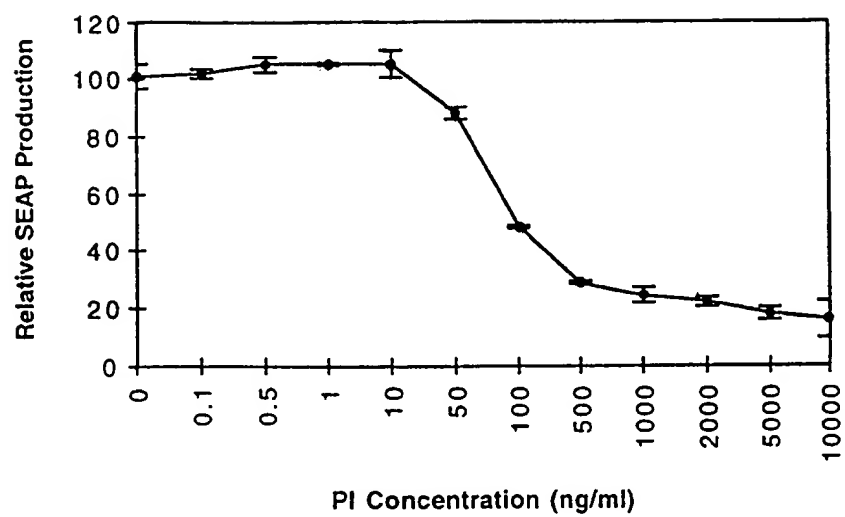
35

A B C   D E F G H   I J K L M

FIG. 1
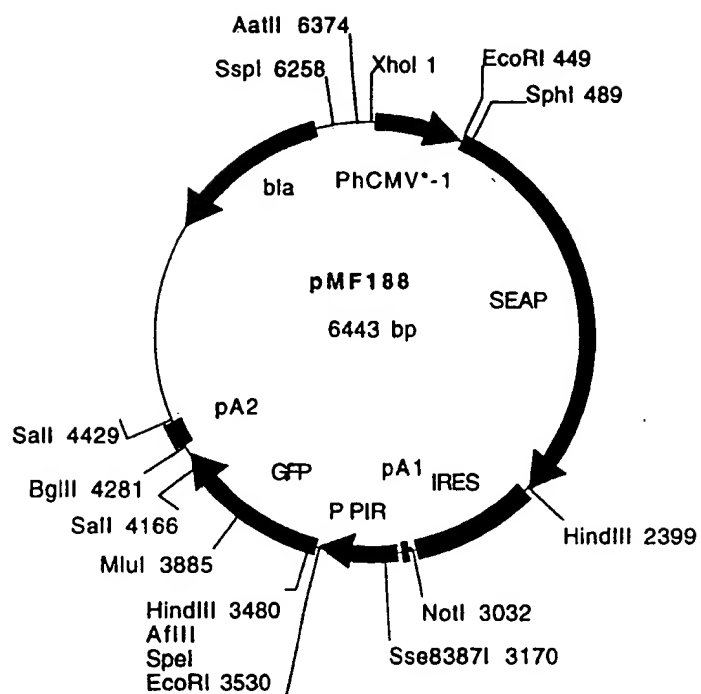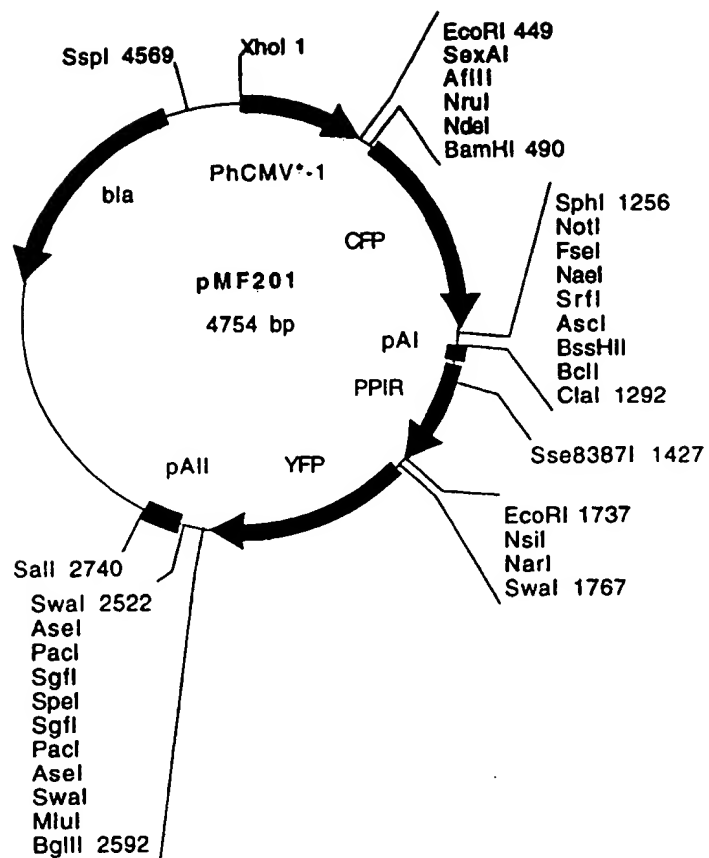
FIG. 2
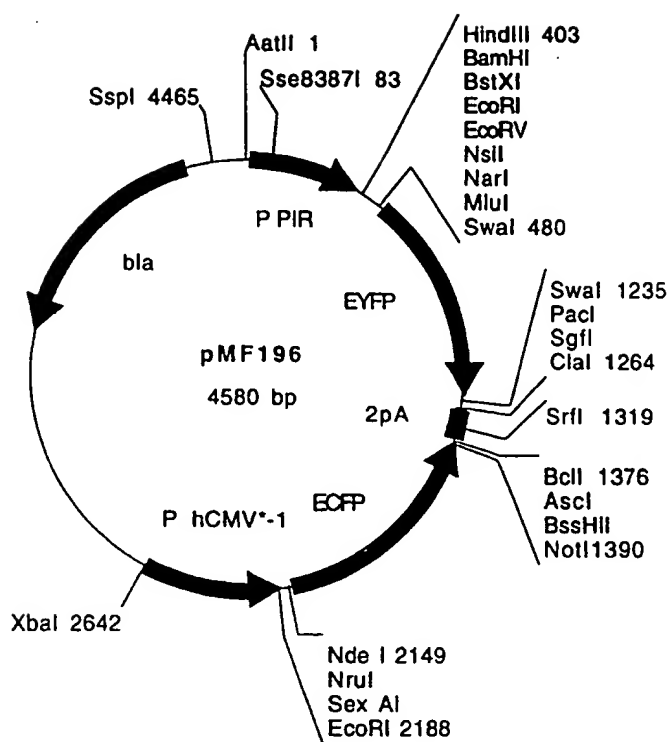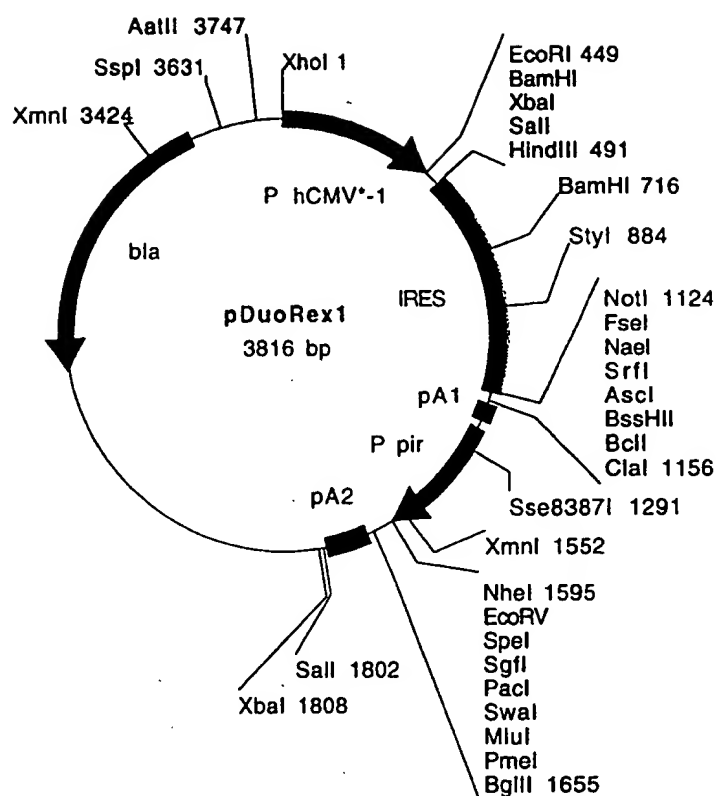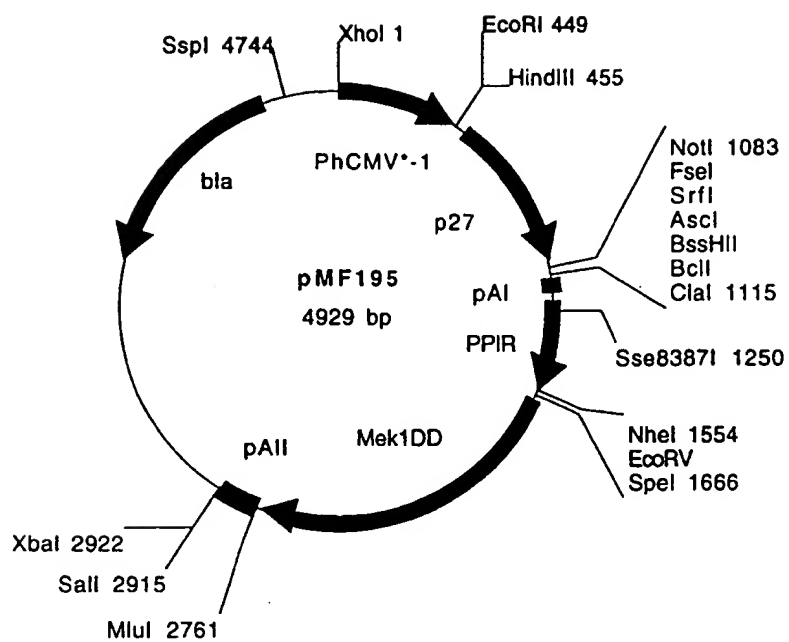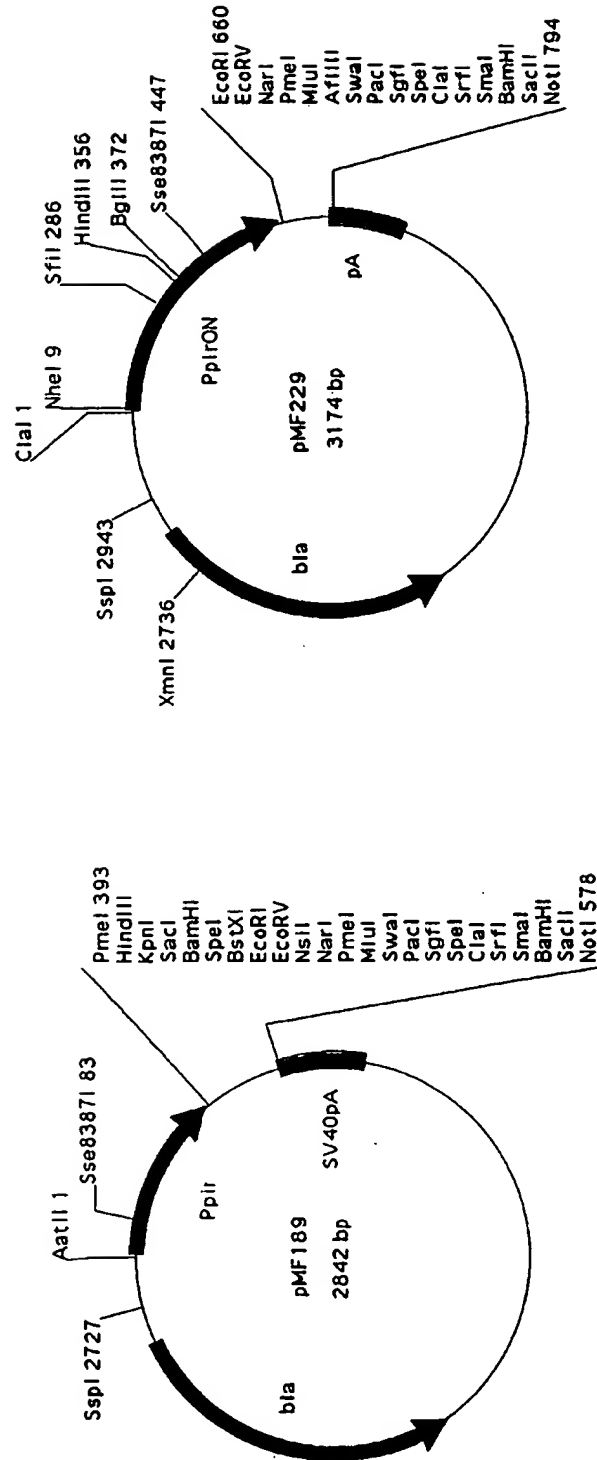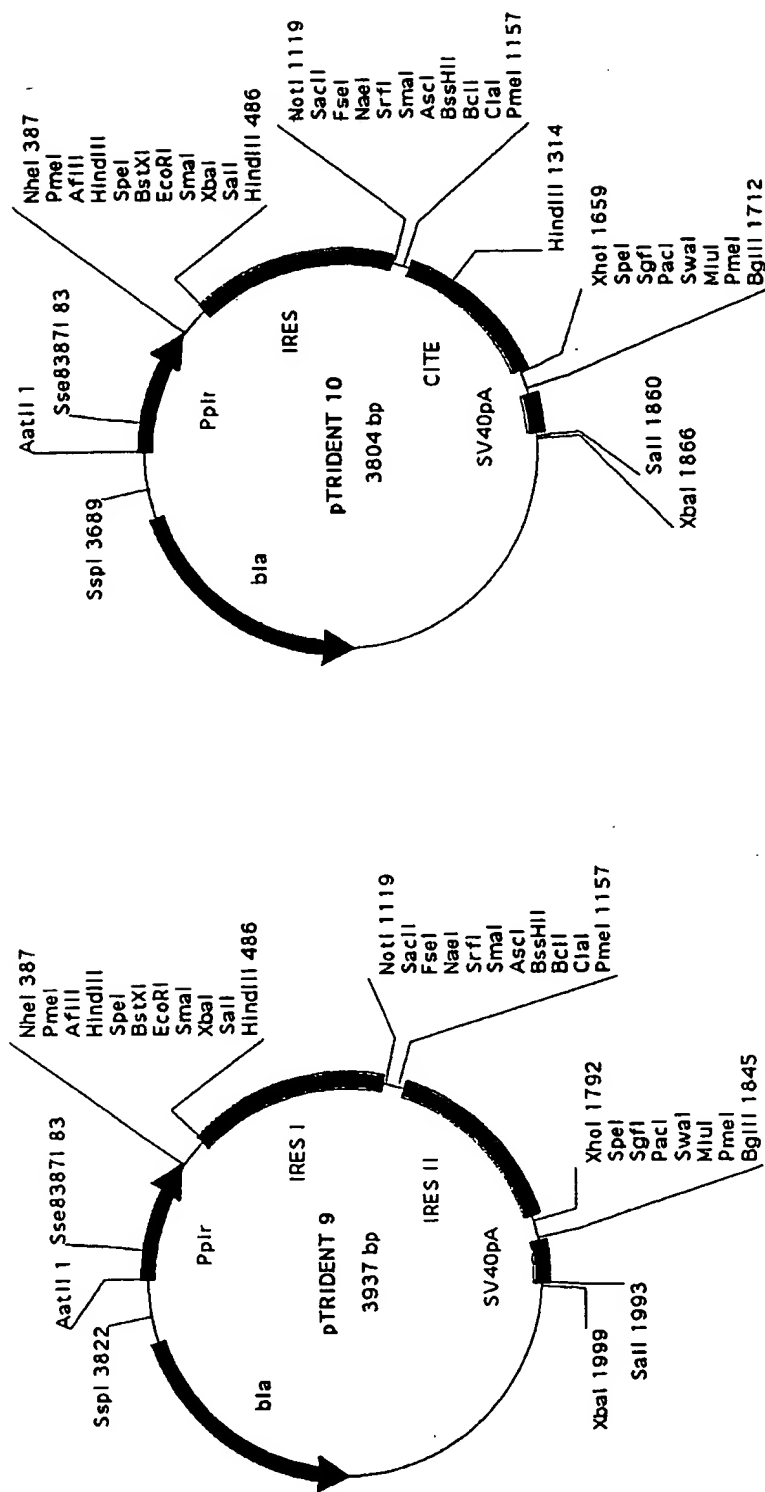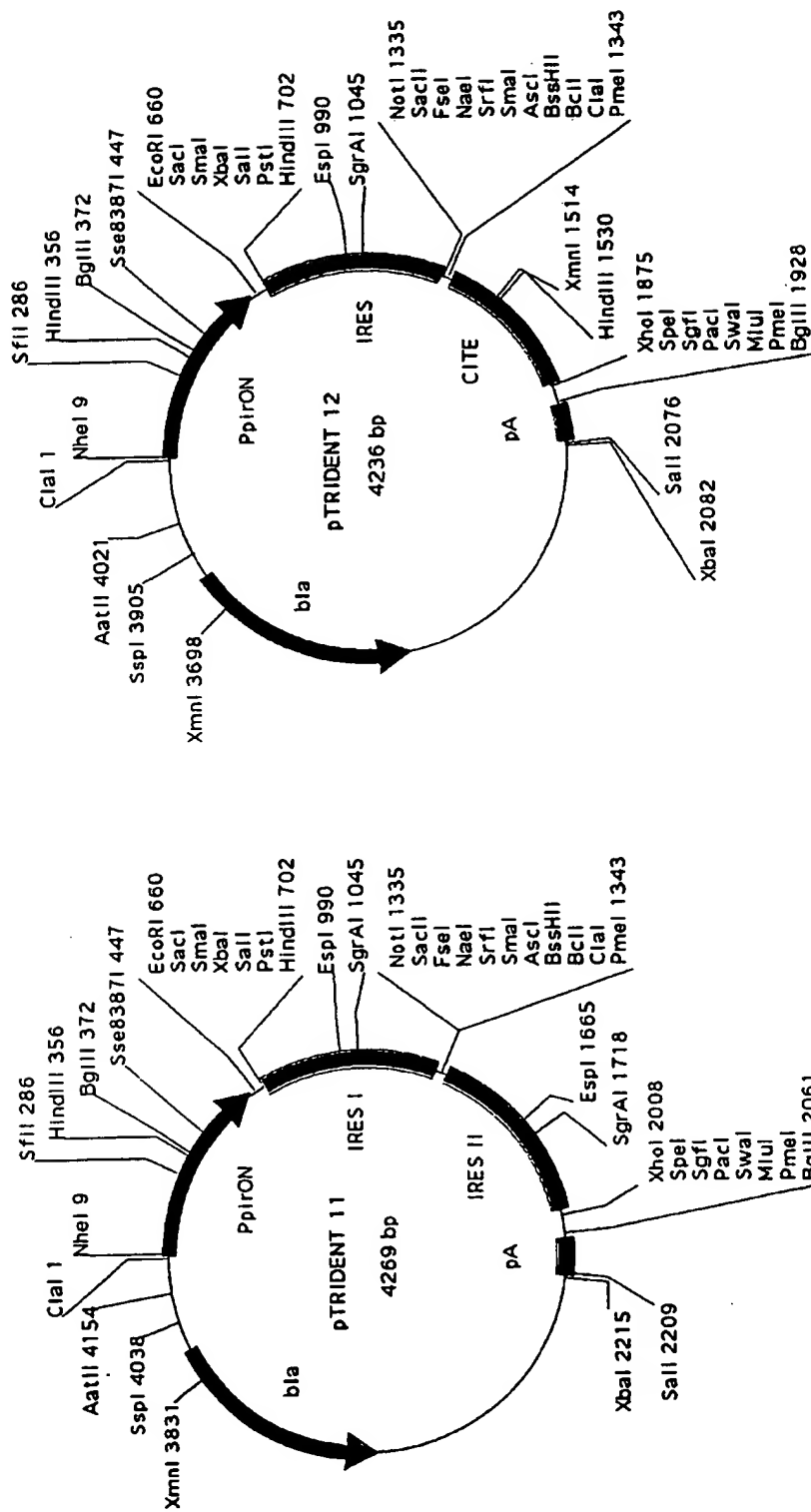
FIG. 3

FIG. 4

FIG. 5

FIG. 6

FIG. 7

FIG. 8

FIG. 9A

FIG. 9B

A.   CLASSIFICATION OF SUBJECT MATTER

IPC(7)   :C12P 21/00; C12N 5/00; C07H 21/04; C07K 14/00
US CL   :435/69.1, 375; 530/350; 536/23.1, 23.4

According to International Patent Classification (IPC) or to both national classification and IPC

B.   FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   435/69.1, 375; 530/350; 536/23.1, 23.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, STN: medline, embase, biosis, caplus
search terms: inventors names, antibiotic, promoter, pristinamycin, virginiamycin, streptogrammin, pip

C.   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y A	US 5,589,362 A (BUJARD et al) 31 December 1996 (31.12.96), see entire document, especially columns 4-5, 9-10, 26-28 and 42-43.	10-28, 33-41 ----- 1-9, 29-32, 42-48
Y A	CLACKSON, T. Controlling mammalian gene expression with small molecules. Current Opinions in Chemical Biology. 1997, Vol. 1, pages 210-218, especially pages 210 and 216.	10-28, 33-41 ------- 1-9, 29-32, 42-48
Y A	GUILFOILE, et al. The Streptomyces glaucescens TcmR protein represses transcription of the divergently oriented tcmR and tcmA genes by binding to an intergenic operator region. Journal of Bacteriology. June 1992, Vol. 174, No. 11, pages 3659-3666, especially Figures 2, 3, 5 and 6.	10-28, 33-41 ----- 1-9, 29-32, 42-48

☐   Further documents are listed in the continuation of Box C.   ☐   See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 JULY 2000	17 AUG 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer Gerald G. Leffers Jr.
Facsimile No. (703) 305-3230	Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet) (July 1998)*